University of Alberta

PATTERNS OF PRENATAL LOSS: IMPLICATIONS FOR PLACENTAL AND FETAL DEVELOPMENT IN THE PIG

by

©

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ABSTRACT

Against a background of changing patterns of prenatal loss in the pig, the implications of increased numbers of embryos *in utero* for placental and fetal development were examined. In the first experiment, laparotomy at day 30 of gestation in gilts, and necropsy of neonatal piglets was used to investigate the relationships between placental size, levels of uterine crowding and fetal development. Although only moderate levels of uterine crowding were observed, a "brain sparing" effect in neonatal piglets was seen in the absence of effects on term placental weight, or birth weight. A second study confirmed high ovulation rates in commercial dam-line sows and established effects of parity on ovulation rate, number of conceptuses *in utero*, placental weight and fetal brain:liver weight ratio (a measure of intrauterine growth retardation, IUGR), as well as an interaction between parity and gestational day on prenatal loss. Further studies utilised surgical oviduct ligation (LIG) to successfully reduce the number of embryos *in utero*. In the third study, purebred sows of parities 4 to 6 underwent either embryo count surgeries at day 30 of gestation or LIG before breeding. In the absence of high ovulation rates in these purebred sows, the threshold for effects of intrauterine crowding on fetal development at day 30 or day 90 were not reached. In the final study, third parity, cross-bred control sows or LIG sows were slaughtered at either day 30 or 90 of gestation and the effects of numbers of conceptuses on prenatal organ and muscle fibre development were determined. The greater number of embryos in control sows exerted negative effects on placental and fetal weights, fetal brain sparing, associated with decreased muscle weights and cross-sectional areas, and a lower total number of secondary muscle fibres. The distribution of myosin heavy chain isoforms did not differ

between groups. Collectively, these studies indicate that the potential for uterine crowding to impact fetal development depends on a complex interaction of a number of factors including parity, genotype and the health status of the sow.

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LIST OF ABBREVIATIONS

AGA	appropriate for gestational age
ANOVA	analysis of variance
bHLH	basic helix-loop-helix
BMPs	bone morphogenic proteins
BS	blocking solution
Ca^{2+}	calcium ion
CG	chorionic gonadotrophin
CHD	coronary heart disease
CL	corpus luteum
CLFS	chronic low frequency stimulation
CSA	cross sectional area
CTR	control sows
CV	coefficient of variance
CVD	cardiovascular disease
CVd%	coefficient of variation of distances
EGF	epidermal growth factor
ELBW	extremely low birth weight
ELISA	enzyme linked immunosorbant assay
F1	first cross generation of two pure breeds
FG	fast twitch glycolytic
FGF	fibroblast growth factor
FGR	fetal growth restriction
FOG	fast twitch oxidative glycolytic
GH	growth hormone
GHR	growth hormone receptor
GLM	general linear model
GLUT-1	glucose transporter protein-1
GP	grand-parent generation (purebred sows)
ICM	inner cell mass
Id	inhibitor of differentiation
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IUGR	intrauterine growth retardation
LBW	low birth weight
LD	*longissimus dorsi* muscle
LDL	low density lipoprotein
LGA	large for gestational age
LH	luteinizing hormone
LIG	ligated sows
LS Mean	least squares mean
MEF2	myocyte-specific enhancer-binding factor 2
MHC	myosin heavy chain
MLC	myosin light chain

MMP	matrix metalloproteinases
MPC	myogenic precursor cells
MRF	muscle regulatory factor
MRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
$PGF_2\alpha$	prostaglandin $F_2\alpha$
Pi	inorganic phosphate
PI3K	phosphoinositide 3-kinase
PKB	protein kinase-B
PKC	protein kinase-C
PL	placental lactogen
PRL	prolactin
PSE	pale soft exudative
pST	porcine somatotrophin
RAP-PCR	ribonucleic acid arbitrarily primed-PCR
Ras-MAPK	Ras-mitogen-activated protein kinase
RIA	radioimmunoassay
RT-PCR	reverse transcriptase – polymerase chain reaction
RTU	real time ultrasound
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGA	small for gestational age
ST	*semitendinosus* muscle
T_3	tri-iodothyronine
T_4	thyroxine
TIMP	tissue inhibitor of metalloproteinases
TGF	transforming growth factor
TH	thyroid hormone
UHO/UHOX	unilateral hysterectomy ovariectomy
VEGF	vascular endothelial growth factor
VLBW	very low birth weight

CHAPTER ONE

INTRODUCTION

Successful commercial pig production depends largely on the productivity of the breeding herd. Strategies used to increase reproductive efficiency include reducing non-productive-days (e.g. herd entry to service interval and weaning to oestrus interval) and increasing the number of pigs weaned per sow per year through attainment of maximum litter size. The number of litters produced per sow on an annual basis is dependent on gestation and lactation lengths and weaning to oestrus interval, whilst number of pigs weaned is dependent on litter size and pre-weaning mortality. In turn, maximum attainable litter size is determined by ovulation rate and littersize actually born is then determined by fertilization rate and embryonic and fetal mortality. Since fertilization rate is widely accepted to be almost 100% under good management conditions, prolificacy is determined mainly by the number of ovulations and by prenatal mortality (van der Lende et al., 1994). The importance of early prenatal mortality (termed embryonic mortality) in determining reproductive efficiency has been recognized for many decades. Numerous studies investigated both the mechanisms involved and the complex range of genetic and environmental factors that influence embryonic mortality and an excellent summary of these factors was presented by Pope (1994).

Traditionally, pre-implantation losses were thought to be the largest proportion of prenatal loss in the pig (as reviewed by Ashworth and Pickard, 1998). The numbers of embryos *in utero* post-implantation were considered to already reflect uterine capacity and, therefore, littersize born. Previous studies of embryo survival have allowed management protocols to be developed that are assumed to maximize litter size born, particularly in first parity females which comprise approximately 40% of the reproductive herd, on the assumption that this would maximize economic returns. However, as initially discussed by Foxcroft (1997) it seems that the patterns of prenatal loss may be changing, with increasing losses occurring during later stages of gestation. The advent of prolific dam-lines currently used in much of the swine industry means that very different animals are now used commercially compared to those used in earlier experiments on prenatal loss, yet even the basic reproductive characteristics of these modern dam-lines have not been reported.

In the pig, ovulation rates increase with increasing parity, and this trend appears to be accentuated in specific dam-line females (Orzechowski, 1998; Vonnahme et al., 2002; Town et al., Chapter 4). Yet, despite very high ovulation rates in some high parity sows, litter size in these sows only increases by 1-2 piglets. Clearly a discrepancy exists between the magnitude of increase in ovulation rate and litter size. As a consequence, Foxcroft (1997) suggested that prenatal survival probably decreases from about 70% at first parity to 50% or less at higher parities. Table 1.1 summarizes data from several experiments, which illustrate the increase in ovulation rate and decrease in embryo survival to day 30 with increasing parity.

Table 1.1 Ovulation and embryo survival rates with increasing parity.

Parity	**Ovulation rate**	**Embryo survival to day 30 (%)**	**Embryo number at day 30**	**Reference**
Gilts	17.1 ± 0.60	83.60 ± 4.30	14	Almeida et al., 2000
1	19.9 ± 1.60	87.50 ± 6.40	17	Zak, 1997
	17.2 ± 0.84	72.72 ± 3.92	13	Foxcroft, 1998 (unpublished)
2+	22.1 ± 0.76	69.03 ± 3.31	15	Patterson et al., 1999 (unpublished)
	26.7 ± 0.77	68.00 ± 2.00	18	Vonnahme et al., 2002

Several studies, including recent work by Almeida et al. (2000), have shown that embryo survival rate can be as high as 100% at day 28 of gestation in gilts. A large range of ovulation and embryo survival rates has been observed in a study by Patterson et al. (1999 unpublished), see Table 1.2.

Table 1.2 Extremes of variation in embryo survival patterns and litter size in parity 2+ animals.

Ovulation rate	Embryo loss to day 30 (%)	Embryo number at day 30	Estimated loss after day 30 (%)	Expected litter size *
30	16.67	25	47.3	10.8
23	26.09	17	21.7	12.0
13	69.23	4	0	15.6

* Estimated litter size based on average litter size of all previous parities

The wide range of variation in embryo survival rate, in addition to the results of Almeida et al. (2000) indicate that a substantial proportion of developing conceptuses are now being lost post-implantation, and more specifically between day 30 and 55 of gestation (Vonnahme et al., 2002). In effect therefore, the earlier peak of prenatal loss driven by competition among embryos in the pre-implantation period (Pope, 1994) is absent in some females and is replaced by a later peak of prenatal loss as uterine capacity becomes an important limiting factor. For example, Père et al. (1997) evaluated the effects of pig embryo number on fetal survival and growth and maternal metabolism, in 114 Large White gilts. Their data supported the hypothesis that uterine capacity limits litter size in sows and that even sows with a normal ovulation rate are affected. Thus, an ovulation rate greater than the number of pigs the sow is able to keep alive until farrowing also results in lighter fetuses at term. Furthermore, existing studies on Intrauterine Growth Retardation (IUGR) also suggest that conclusions based only on a consideration of fetal weight will overlook critical effects on fetal development that are established early in gestation. In a study of the association between within-litter differences in prenatal development and postnatal survival and growth, van der Lende and de Jager (1991) concluded that the lower pre-weaning growth of the disadvantaged pigs born could not entirely be explained on the basis of their lower birth weight. This suggests that IUGR has a more complex effect on the developmental potential of such pigs.

The existence and pathology of "runted" or IUGR offspring has been thoroughly described in the pig (Adams, 1971; Widdowson, 1971; Cooper et al., 1978; Hegarty and

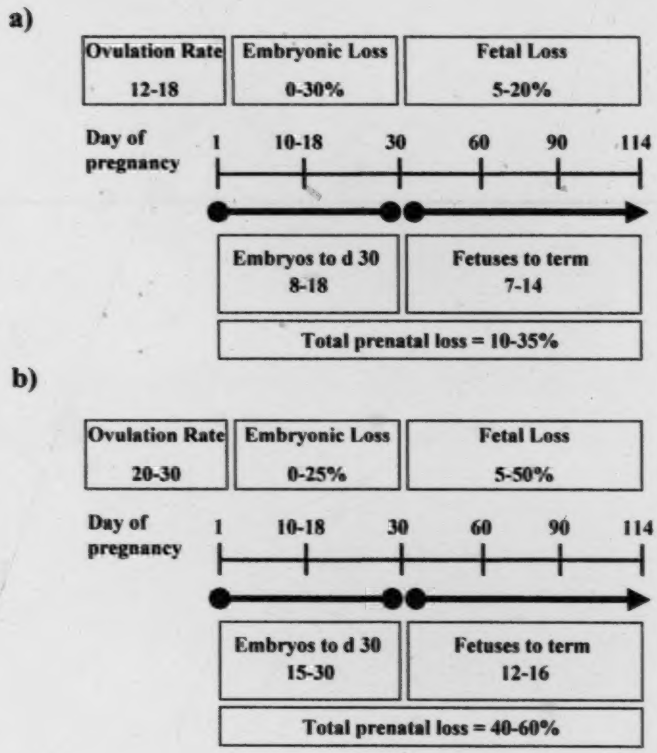
Allen, 1978; Flecknell et al., 1981). A common finding is that the brain is the organ least affected by growth retardation, and the brain:liver weight ratio can be used as an effective measure of IUGR (Bauer et al., 1998). Studies of within-litter variation in prenatal development suggested that the extremes of IUGR were identified within a discrete subpopulation of fetuses (Royston et al., 1982; Wooton et al., 1983). Crowding of the uterus in the early period of gestation due to changes in the levels of prenatal loss potentially affects fetal development of the remaining conceptuses in a manner analogous to IUGR, raising important questions for fetal and postnatal development, particularly with respect to the development of the fetal muscle fibres, such effects are analogous to those seen in nutritionally-challenged sows (Dwyer et al., 1994). Alternatively changes in placental efficiency (Biensen et al., 1998) could compensate for earlier effects of uterine crowding on placental size and essentially increase uterine capacity, as reported in prolific Meishan females (Ford, 1997).

The placenta has been an organ of interest for several centuries, since it plays a critical role in providing the interface between mother and fetus as the site of exchange of nutrients and waste products. Adequate placental function is, therefore, crucial for normal fetal growth and development. Therefore, changes in placental function exert negative effects on embryonic and fetal development, this may also have long term postnatal effects on the body's structure, physiology and metabolism. This has important implications for the survival and well being of all mammals. The effect of placental insufficiency on fetal skeletal muscle growth is particularly important in the agricultural industry, as total muscle mass and rate of muscle growth are important factors in meat production.

In a commercial context, in addition to achieving acceptable production in terms of pigs weaned per sow per year, the size and growth uniformity of pigs weaned is critical for efficient use of "All-In/All-Out" nursery and grow/finish facilities, and for effective marketing to meet specific carcass specifications. Pork producers are under increasing pressure to deliver uniform carcasses to the packer, following a tightly controlled time schedule. Therefore, decreased variation in birth weight and optimized lean growth performance of all piglets within a nursery would have enormous economic benefits to the industry.

Against a background of changing patterns of prenatal loss in the pig (see Figure 1.1), the work undertaken in this thesis examines the implications of increased numbers of embryos *in utero* for placental and fetal development. Parity and genotypic influences are considered and thresholds for effects of uterine crowding on fetal development are identified. The second chapter is an in-depth review of the literature, examining the pertinent factors relating to patterns of prenatal loss and the effects on placental and fetal development. Due to the vast amount of available literature, the review is focused on the pig, although where appropriate, studies in other livestock species or humans are discussed. The first section of the literature review provides a general background to placental anatomy and function, focusing in particular on the placental type seen in the pig. Events occurring after fertilization through to the development of the placental membranes are discussed. The endocrine regulation of prenatal growth and the impact of placental-fetal endocrine interactions on fetal and neonatal outcome are explored. The second section reviews the patterns of embryonic loss during gestation and addresses factors that influence placental and embryonic growth, with a particular focus on the detrimental effects of uterine crowding on development. The implications of placental function for fetal survival, development, the incidence of intrauterine growth restriction, the link to retarded postnatal growth and the occurrence of diseases in adulthood are discussed. The final section addresses the pattern of pre- and postnatal muscle development in the pig, including the regulatory factors involved in myogenesis and the relationships between muscle fibre type and number, and postnatal growth potential.

Figure 1.1 a) Traditional view of the determinants of litter size, still applicable to gilts and primiparous sows. b) Revised view, particularly applicable to high parity prolific sows.


a)
Ovulation Rate
12-18
Embryonic Loss
0-30%
Fetal Loss
5-20%
Day of pregnancy
1
10-18
30
60
90
114
Embryos to d 30
8-18
Fetuses to term
7-14
Total prenatal loss = 10-35%
b)
Ovulation Rate
20-30
Embryonic Loss
0-25%
Fetal Loss
5-50%
Day of pregnancy
1
10-18
30
60
90
114
Embryos to d 30
15-30
Fetuses to term
12-16
Total prenatal loss = 40-60%

The four studies undertaken as part of this doctoral research program are presented as sequential chapters (3-6), comprising extended versions of papers submitted for journal publication. The first study (Chapter 3) used midline laparotomy surgery at day 30 of gestation, umbilical tagging at farrowing and necropsy of neonatal piglets to investigate the relationships between placental size, levels of uterine crowding and various aspects of neonatal development in gilts. Moderate levels of uterine crowding were observed which resulted in a "brain sparing" effect in neonatal piglets in the absence of effects on birth weight.

In the study described in Chapter 4, collaboration with Swine Graphics Enterprises Inc (Webster City, IA) provided the opportunity to collect extensive information from commercial dam-line sows during depopulation of an entire breeding unit. Pregnant sows were divided into three parity groups and were slaughtered at three time points during gestation. These data were used to examine the relationship between ovulation rate and prenatal losses and to extend previous studies to determine associations between the pattern of prenatal loss and placental and fetal development. A significant effect of parity on ovulation rate and the number of conceptuses *in utero*, placental weight and fetal brain:liver weight ratio (a measure of IUGR) as well as an interaction between parity and gestational day on prenatal loss was established. The results of the data accumulated from this study of commercial culled sows, encouraged us to develop appropriate experimental paradigms for studying direct effects of the pattern of prenatal loss on fetal development and particularly muscle fibre development within the swine herd at the University of Alberta (Chapters 5 and 6).

Chapter 5 describes a study using mature purebred sows of parities 4-6 which underwent either embryo count surgeries at day 30 of gestation or were subjected to unilateral oviduct ligation (LIG) before breeding. Animals were slaughtered at day 30 or day 90 for collection of reproductive tract data and fetal necropsies. Ligation surgeries successfully reduced ovulation rates available for fertilization, however, in the absence of high ovulation rates in these purebred sows, the threshold for effects of intrauterine crowding on fetal development did not appear to have been reached.

The final study is described in Chapter 6. Unmodified, third parity, control sows (CTR) or sows subjected to unilateral oviduct ligation before breeding were slaughtered at either day 30 or 90 of gestation and used to determine the effects of numbers of conceptuses *in utero* on prenatal, and particularly muscle fibre development. Again, oviduct ligation reduced the number of viable embryos at day 30 and fetuses at day 90. A wider range of number of embryos *in utero* in this study, revealed negative effects on placental and fetal weights in CTR sows. Necropsy results demonstrated effects of brain sparing and IUGR in fetuses from CTR sows. Furthermore, in CTR fetuses, muscle weights and cross-sectional areas were lower and immunohistochemical analysis revealed the total number of secondary fibres was also lower in CTR fetuses. The distribution of myosin heavy chain (MHC) -Iβ, -IIa, fetal and embryonic isoforms examined by Western blotting and SDS PAGE did not differ between groups. Thus, the results of Chapter 6 showed that even the relatively modest uterine crowding occurring naturally in CTR sows, negatively affected placental and fetal development, and the number of secondary muscle fibres.

Collectively, these studies indicate that the potential for uterine crowding to impact fetal development to varying degrees depends on a complex interaction of a number of factors including parity, genotype and the health status of the sow. The implications of the experimental designs and the overall results are discussed in the final chapter of this thesis (Chapter 7). Future studies that could be implemented to further define thresholds and examine the consequences of more extreme crowding *in utero* on pre- and postnatal development, resulting from the changing patterns of embryonic survival are discussed.

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CHAPTER TWO

LITERATURE REVIEW

2.1 The pattern of placental development and the endocrine regulation of placental and prenatal growth

The first section of this review of the literature provides an understanding of events that occur during early pregnancy in terms of placental and fetal development. Knowledge of placental structure and function is reviewed in the context of nutrient transport and endocrine regulation of prenatal growth. This provides the background for later sections detailing mechanisms by which normal prenatal development may be affected, with implications for prenatal survival, fetal growth and development, and longer term postnatal outcome. A particular focus on fetal muscle development reflects the importance of muscle tissue in a commercial, meat-producing animal like the pig.

2.1.1 Placental structure and classification

The mammalian placenta is an organ formed by the apposition of maternally and embryonically derived tissues inside the uterus. Placental tissue is highly vascularized by maternal and fetal vessels, which due to their close proximity allow the diffusion of materials between the maternal and fetal blood. The placenta plays a critical role in providing an environment that supports optimal fetal growth. It provides the site of exchange of materials between mother and young, transferring nutrients and oxygen from mother to fetus, and waste products in the opposite direction. Nutrients may be modified by metabolic activities of the placenta, either for consumption within the placenta or for release to the fetus. The placenta maintains an immunological barrier protecting the fetus from pathogens and from rejection by the maternal immune system. The placenta is also an active endocrine organ capable of synthesizing and secreting a wide range of protein and steroid hormones, growth factors, cytokines and other bioactive molecules.

Placental structure has been an ongoing area of research for centuries. In the early 16th Century, Leonardo da Vinci made drawings of the human uterus with a fetus *in situ* in which the rim of the placenta and the umbilical cord can clearly be seen. In 1774, the work of the Englishmen, John and William Hunter, set new directions in the understanding of the placenta as a nutritive and excretory pathway for feto-maternal exchange. Their work on the human placenta showed the presence of maternal blood in the intervillous space and demonstrated the curling arteries of the endometrium (Ramsey, 1977). Placentae can be classified by gross morphology based on a system introduced by Fabricius in the 17th Century. There are four main types of mammalian placenta based on the appearance of the sites of chorionic attachment to the endometrium. These are known as diffuse, cotyledonary, zonary and discoidal. The diffuse placenta is the type that occurs in the pig, however, the other types of placenta will also be briefly described.

Placentae can also be classified histologically, based on the number of cell layers separating maternal and fetal blood (Figure 2.1). The first placental classification of this type was put forward by Grosser in 1909, as will be discussed in section 2.1.1.2. It is currently accepted that there are three main classes of placenta - haemochorial, endotheliochorial and epitheliochorial, although some variation exists within each class (as reviewed by Renfree, 1985). The pig placenta is epitheliochorial.

Mossman (1937) reviewed current knowledge of embryology and placentology. He defined the placenta as "an intimate apposition or fusion of the fetal organs to the maternal (or paternal) tissues for physiological exchange". Later, Amoroso (1952) covered all the known aspects of placentology in Marshall's Physiology of Reproduction. Since then, research in placental function and its role in the control of fetal growth has been extensive.

2.1.1.1 Morphological classification

The cotyledonary placenta is characteristic of ruminants. The chorion has specialized villi restricted to circular or oval areas covering the surface of the chorionic sac. These specialized clumps of chorionic villi are known as cotyledons. The cotyledons only develop in areas of the placenta that overlie regions of the endometrium known as caruncles. The fetal cotyledon and the maternal caruncle together form a unit called the placentome. Placentome number varies widely between species from as few as three or four per uterine horn in the deer up to 180 in the goat and giraffe. The placentomes are the only areas of true placental attachment to the endometrium and as such are the only sites of feto-maternal exchange. The zonary placenta is characteristic of the carnivores. In this type of placenta, chorionic villi aggregate to form a broad band around the "equator" of the chorionic sac (like a girdle). The band may be complete as found in the dog and the cat, or it may be incomplete as in ferrets, seals and bears. The zonary placenta always has a central or marginal effusion of maternal blood, which is called the hemophagus organ. The discoidal placenta is found in a diverse group of mammals including man, many species of non-human primates, rodents such as the rat, mouse and guinea-pig and in the lagomorphs such as the rabbit. The chorionic villi are arranged in a circular plate, which may be single (as in man) or double discoid (as in Macaque monkeys).

As mentioned previously, the diffuse placenta is the type of placenta found in the pig. It also occurs in the horse, camel, dolphin and whale. In the diffuse placenta, the microvilli of the chorion are distributed more or less evenly over the entire surface of the chorionic sac. Placental microvilli interdigitate with corresponding depressions (or villi) in the uterine epithelium. Feto-maternal exchange therefore takes place over nearly the entire surface of the chorion.

2.1.1.2 Microstructure of placental layers

Grosser systematically classify placentae into different groups as long ago as 1909. His classification system still survives today although it has been modified and refined with the emergence of new technologies - in particular the electron microscope. Placentae are classified histologically, based on the number of cellular layers separating maternal and fetal blood. Grosser's concept was that the efficiency of the placenta is inversely proportional to the thickness of the barrier (or the number of cellular layers) separating the maternal and fetal circulation. He suggested four different types of placenta existed (epitheliochorial, syndesmochorial, endotheliochorial and haemochorial). This functional concept has since been largely disproved but the classification has remained useful for categorizing placental types - three main placental classes are now accepted although variation exists within each class depending on species.

Haemochorial placentae such as those found in the human are composed of three cellular layers separating maternal and fetal blood. The fetal capillary endothelium, fetal connective tissue and fetal chorionic epithelium comprise the placental barrier. Maternal blood directly bathes the outermost trophoblast layer (the chorionic epithelium). Endotheliochorial placentae have four cellular layers separating the maternal and fetal circulation. In addition to the three fetal layers previously mentioned, the maternal capillary endothelium is an additional layer. This type of placental microstructure is found in carnivores such as the dog, cat, bear and mink. The epitheliochorial placenta, as found in the pig, is composed of six cellular layers (Figure 2.1). The fetal capillary endothelium, the fetal connective tissue, the fetal epithelium which is in apposition with the maternal endometrial epithelium, the maternal connective tissue and finally the maternal capillary endothelium.

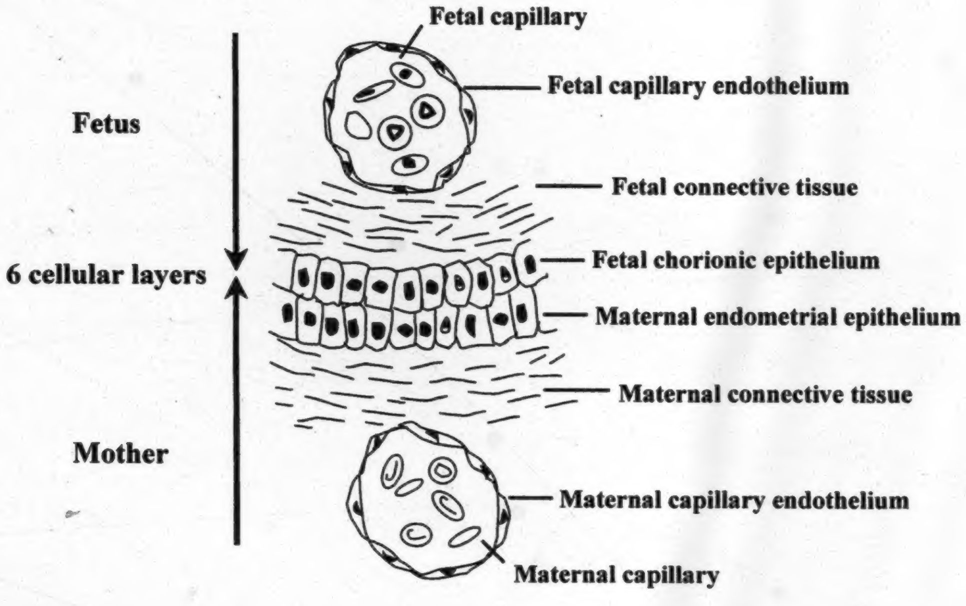

Fetal capillary
Fetal capillary endothelium
Fetus
Fetal connective tissue
Fetal chorionic epithelium
6 cellular layers
Maternal endometrial epithelium
Maternal connective tissue
Mother
Maternal capillary endothelium
Maternal capillary

Figure 2.1 Microstructure of the epitheliochorial placenta (adapted from Renfree, 1985)

2.1.2 Summary of fertilization, attachment and placentation in the pig

Perry (1981) and Stroband and Van Der Lende (1990) both presented excellent reviews of the events that occur in the female reproductive tract following fertilization through to the formation of the placental membranes. In the pig, fertilization occurs in the ampulla of the oviduct, near the ampullary-isthmic junction (Hunter, 1977). Two to three days after fertilization the embryos enter the uterus, and by this time, they have reached the 2-4 cell stage. The 8-16 cell morula stage is reached around day 4. Within the solid ball of cells that comprises the morula, a fluid filled cavity appears called the blastocoele. The blastocoele enlarges rapidly until the embryo resembles a hollow sphere, which is termed the blastocyst. Blastocyst formation is characterized by a change in trophoblast cell shape from spherical to wedge shape. Cells flatten against each other so as to maximize cell to cell contact and special links or junctional complexes develop between them. The whole process is termed compaction and gives the cells polarity for the first time. The blastocyst is composed of a single peripheral layer of large flattened cells (the trophoblast or trophectoderm) with a clump of smaller cells to one side of the central cavity. The cluster of cells is called the inner cell mass (ICM) which gives rise to the embryo, while the trophoblast goes on to form the placenta and embryonic membranes.

2.1.2.1 Blastocyst hatching

Blastocysts are initially still surrounded by the zona pellucida from which they hatch between days 6 and 7 of pregnancy. The zona pellucida is a transparent, tough and elastic mucopolysaccharide coat (Perry, 1981) which functions to hold the cleaving embryo together during its passage through the oviduct and prevents it from sticking to the oviductal wall. The factors causing zona lysis are unknown but embryonic enzymes, mechanical mechanisms or uterine factors may be involved (Stroband and Van der Lende, 1990).

After hatching until days 11-12, the spherical blastocysts remain free in the uterine lumen. Their diameter increases up to 10mm during this period. Between days 7 and 12 after fertilization, the embryos initially migrate from the oviductal towards the cervical end of the uterine horns as a consequence of peristaltic movements of the uterine wall (Perry, 1981).

2.1.2.2 Spacing of embryos

As part of the spacing process, embryos may migrate completely from one side of the uterus to the other. This trans-uterine migration was neatly demonstrated by an experiment carried out by Dziuk et al. (1964), who transferred eggs from a black breed of pigs into the left uterine horn and eggs from a white breed into the right uterine horn of gilts. When the gilts were killed at 90 days of gestation, the extent of internal migration of embryos could be clearly seen by the colour of the fetuses (Figure 2.2).

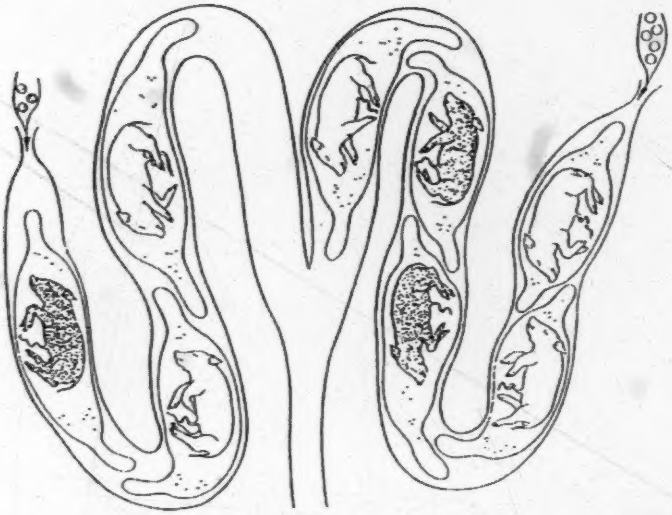


Figure 2.2 Example of intrauterine migration and mixing of embryos (from McLaren, 1985).

The equidistant spacing that occurs between porcine embryos indicates that spacing is not entirely passive but that the embryos can exert a repelling effect on each other (Dziuk, 1985). Rahima and Bruce (1986) also concluded that an active mechanism promotes even spacing of conceptuses in the rat. They describe the evenness of spacing in the uterine horn in terms of the coefficient of variation of distances (CVd%) between adjacent conceptuses and compared that against the expected CVd% for conceptuses located randomly within the uterine horn. The observed evenness of spacing was found to be substantially greater than could be expected from a completely random distribution.

Even spacing of conceptuses is likely to be important to provide maximum room for fetal growth and to allow establishment of each placental unit with minimum competition from adjacent placentae for available maternal blood vessels. Any factor causing uneven spacing may limit placental growth and in turn retard fetal growth. Wellstead et al. (1989) tested this hypothesis by administering the prostaglandin inhibitor indomethacin on day 5 of gestation in the rat, which caused random unevenness of spacing, suggesting that prostaglandins have a role in the control of spacing. However, evenness of spacing was not totally disturbed, so it would seem that the indomethacin administered did not completely block the action of prostaglandins. There was no evidence that the level of local crowding produced by indomethacin administration influenced growth of individual fetuses or placentas. However, indomethacin had a general effect on fetal and placental growth causing initial growth retardation. The authors concluded that the anatomy of the maternal arterial supply to the uterus has sufficient reserve to cope with relatively uneven spacing of fetuses in the rat.

The mechanism by which polytocous species position their conceptuses evenly within the cavities of the uterine horns remains unclear although several hypotheses have been put forward. Dziuk (1985) described the effects of the location of the porcine embryo within the uterus on pregnancy and fetal survival. He suggested that an embryo could create space around itself by promoting uterine contractility originating from the location of the embryo, which would tend to oppose contractility in adjacent portions of the uterus and hence prevent the migration of embryos towards each other. If several embryos in the same uterus were each generating such repelling contractions, embryos would move to equal distances from each other.

Another possibility is that the conceptuses are secreting substances (for example extracellular signaling proteins or cytokines that are involved in cell-cell communication). These substances may act to signal the position of an embryo to its neighbour and thereby prevent adjacent embryos from attaching at too close proximity or unequal distances apart. From my search of the literature, it seems that the factors responsible for the uniform spacing of porcine embryos throughout the uterus remain to be elucidated. However, a large amount of literature exists showing evidence of the involvement of peptide growth factors in the initiation, establishment and maintenance of pregnancy, and in maternal-embryonic communication both in the pig and in other species such as ruminants and rodents. Some of this information will be covered in later sections.

Whatever the signaling mechanism for equidistant spacing of porcine embryos may be, it clearly has to occur during the window of time before final attachment of the embryos to the uterine epithelium. Dhindsa et al. (1967) determined that migration of embryos from one horn to the other usually occurred first on day 8 or 9 and the uterus was occupied completely by day 15.

2.1.2.3 Elongation and attachment

Porcine embryos start to elongate around day 12, mainly due to reorganization of the cells (cell hyperplasia occurs later). Blastocysts undergo a reduction in diameter resulting in the formation of filamentous structures of up to 100cm long. Elongation does not occur at the same moment in all conceptuses due to variation in developmental stage (Stroband and Van Der Lende, 1990). The regulation of the elongation process and the genes associated with these events are unknown. However, Wilson et al. (2000) have begun the characterization of differential gene expression by trophectodermal cells during elongation of the porcine conceptus using RNA arbitrarily primed-PCR (RAP-PCR).

At the same time as elongation occurs, embryos start to synthesize and secrete oestrogens (as reviewed by Geisert et al., 1990; Bazer et al., 1991), which are crucial for the maternal recognition of pregnancy in the pig and the maintenance of pregnancy. Oestrogens are important for the maintenance of the corpora lutea and hence the continuation of progesterone secretion. Embryonic oestrogens are also important because they stimulate the secretion of proteins from the endometrium. The amount of oestradiol-

17β synthesized and secreted by pre-implantation conceptuses has been positively associated with the endometrial secretion of growth factors, including IGF-I (Wilson and Ford, 1997). The role of IGF-I in fetal and placental growth will be discussed in section 2.1.4.3.1. The effect of oestradiol-17β during the time of conceptus elongation on placental size at term in Meishan pigs was examined by Wilson and Ford (2000). The administration of oestradiol-17β around the time of conceptus elongation increased placental size and led to a reduced placental efficiency.

Dziuk (1985) notes that in the pig, the distribution of embryos is critical to the maintenance of pregnancy. A significant section of unoccupied uterus at day 12 will prevent continuance of pregnancy regardless of the number of embryos present in the occupied section. When the number of embryos is so few as to not occupy the uterus fully, pregnancy will not continue. A minimum of 4 embryos is generally thought to be required at day 12 to maintain pregnancy by signaling to the maternal system. Removal of embryos from a significant section of the uterus after day 14 was shown not to stop an existing pregnancy - highlighting that the critical time of maternal recognition of pregnancy occurs around day 12.

Trophoblast invasion and erosion of the uterine epithelium does not occur in the pig. Attachment starts around day 13-14 and begins with loose contact between trophoblast and uterine membranes near the embryoblast and is completed by intermingling of uterine and trophoblastic microvilli after day 18. Attachment constitutes the beginnings of the formation of the placental membranes. The elongated blastocysts follow the endometrial folds. Each blastocyst occupies only a relatively short length of the uterus. Wigmore and Stickland (1985) describe the growth of the porcine placenta from 38 days until term. Placental area increased during the period of study due to increases in the uterine circumference at the sites of conceptuses. No change was found in uterine horn length or placental length with age. Placental length was shorter in more crowded horns and showed a U-shaped distribution with position within uterine horns (the longest placentae occurring at the ovarian ends of the uterine horns).

2.1.2.4 Fetal membrane development

The fetal membranes are derived from three basic extra-embryonic germ layers (the ecto-, meso- and endoderm). The single layered trophoblast (or trophectoderm) is the first extra-embryonic membrane to differentiate (Stroband and Van der Lende, 1990; Perry, 1981). As a result of amniogenesis, the trophoblast layer differentiates to become the chorion and is invested with a layer of avascular mesodermal cells. This outer envelope encloses the entire embryo and the other three extra-embryonic membranes, the amnion, yolk sac and allantois.

In species like the pig, the amnion comes to surround the embryo completely as a result of folding and fusion of the trophoblast. The amnion provides a fluid filled environment in which the embryo can float and develop in a state of weightlessness, thus allowing symmetrical development of embryonic tissues and appendages without pressure and trauma from surrounding structures. In addition to protecting the embryo against mechanical shock, amniotic fluid also provides protection from desiccation. The amnion is never vascularized.

The yolk sac occurs in all embryos with an amnion and in mammals it develops early from the blastocoele cavity. After fusion with the chorion distal to the embryo, the yolk sac may become vascular (trilaminar) or avascular (bilaminar). The choriovitelline placenta thus formed may constitute the first functional attachment - providing a site of exchange with the mother. (In some mammals - the human for example, the yolk sac becomes vestigeal after a week or so. In others, including the rabbit, the yolk sac remains an important site of nutrient and antibody exchange throughout the pregnancy. In the pig, the yolk sac reaches its maximum development around day 18 and after its isolation from the trophoblast by the expansion of the exocoele the yolk sac rapidly shrinks, becoming relatively inconspicuous by day 20 (Perry, 1981).

The allantois is an outgrowth of the endodermal lining of the embryonic gut, receives fetal urine and eventually forms part of the urinary bladder. The allantois is derived from endoderm and vascular mesoderm and as it expands into the exocoele it acquires an outer layer of vascular mesoderm. Ultimately, the vascular allantois fuses with the chorion to form the definitive allanto-chorionic placenta of birds, reptiles and mammals. In the pig, as in most mammals, the allanto-chorion takes over from the chorio-vitelline placenta as the main organ of nutritive and respiratory exchange.

Moritz and Wintour (1999) reviewed the functional development of the embryonic and fetal renal organs in mammals. Briefly, there are three different pairs of renal organs during development. The pronephros and mesonephros exist for defined periods of intrauterine development. These organs then regress and the third organ, the metanephros, becomes the permanent adult kidney. The major role of the metanephros whilst *in utero* is to produce relatively large volumes of hypotonic urine, which is essential for the maintenance of amniotic and allantoic fluid volumes vital for normal placentation and development.

2.1.2.5 Placental fluid volume and composition

Knight et al. (1977) characterized placental fluid volume changes throughout gestation in the pig. A rapid increase in allantoic fluid volume was noted between days 20 and 30 of gestation (Figure 2.3a) and was associated with initial expansion of the allanto-chorionic membranes and establishment of intimate contact between the placenta and endometrial surface. At day 30 of gestation, allantoic fluid volume was correlated with placental weight, length and allantoic fluid oestrone concentration. The authors discussed the fact that mechanisms responsible for water accumulation in the allantoic cavity were not known, nor whether estrogens may influence the process. Allantoic fluid volume fell between days 30 and 40 and the final period of fluid accumulation occurred between days 40 and 60 of pregnancy, when volume was again significantly correlated with placental length and weight. Measurable amounts of amniotic fluid were not present prior to day 30 of gestation. Amniotic fluid then increased in volume from day 30 to 70, remained stable to day 80, and then decreased to term (Figure 2.3b).

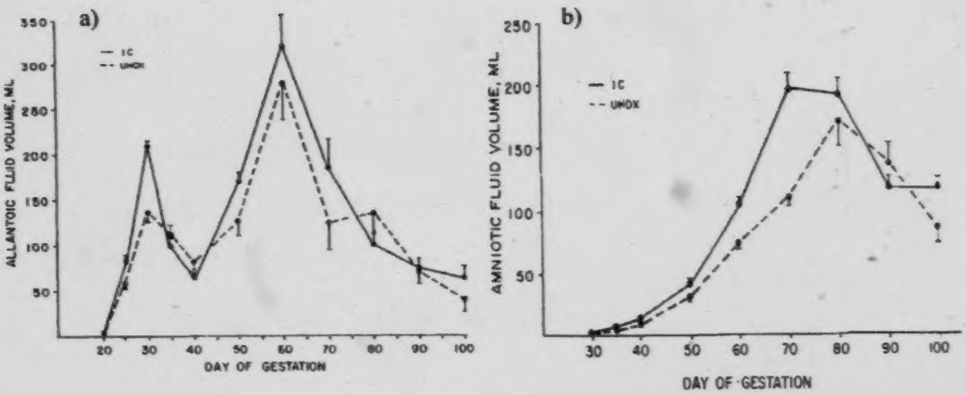


Figure 2.3 Changes in a) allantoic fluid volume and b) amniotic fluid volume/conceptus for intact control (IC) (x ± SEM) and unilaterally hysterectomized-ovariectomized (UHOX) (x ± SEM) at various stages of gestation (adapted from Knight et al.,1977).

The dynamic changes in fetal fluid volumes observed by Knight et al. (1977) were confirmed by Goldstein et al. (1980), who also determined changes in electrolyte composition and osmolarity. A marked difference in composition of allantoic and amniotic fluid led to the conclusion that compositional differences, in addition to the differing patterns of fluid volume changes, indicate that the origin of the two fluids may be different. The contribution of one fluid pool to the other is not known. The composition of fetal urine, stomach fluid and yolk sac fluid were analyzed and results indicated that these fluids could not be a major source of allantoic and amniotic fluids, especially early in gestation. Additionally, a comparison of electrolyte concentrations of maternal plasma and allantoic fluid suggested that allantoic fluid is not a dialysate of serum. It was suggested that the changes in ion composition of the fetal fluids may be critical for development of the fetus.

Although traditionally considered as the reservoir of fetal wastes, the role of the allantoic sac in fetal nutrition was more recently highlighted by Wu et al. (1996) who noted the unusual abundance of the amino acids arginine and ornithine in porcine allantoic fluid. Ornithine is the precursor of polyamines, which are essential to early mammalian embryogenesis, and arginine is the precursor of ornithine and nitric oxide, a free radical with a possible role in regulating uterine and placental-fetal blood flow during pregnancy (as reviewed by Wu et al., 1996).

2.1.2.6 Fetal development and growth of the uterus

Wu et al. (1987, 1988a) compared uterine length, weight and diameter between gravid and non-gravid horns within unilaterally pregnant gilts to determine the influence of embryos on growth of the uterus. They concluded that as gravid horns were 40% longer than non-gravid horns between day 18 and 27 of gestation, embryos first influence the growth pattern of the uterus at day 18 of gestation.

2.1.3 Placental nutrient transport

The placenta mediates the active transport of nutrients and metabolic wastes across the barrier separating maternal and fetal compartments, as well as modifying the composition of some nutrients through its own metabolic activity. Placental transport of nutrients has been the subject of reviews by Hay (1994) and Garnica and Chan (1996). Père (2003) reviewed differences in materno-fetal exchanges and nutrient utilization between species. The exchange of nutrients between the placenta and fetus involves three major mechanisms. Firstly, direct transfer of nutrients from the maternal to fetal plasma, secondly placental metabolism and nutrient consumption, and thirdly placental metabolism of nutrient substrates to alternate substrate forms.

Glucose is the principle carbohydrate transported to the fetus by stereospecific facilitative transporter proteins (as reviewed by Hay, 1994; Garnica and Chan, 1996). GLUT 1 is the predominant glucose transporter although other isoforms include GLUT 3 and 4. In all species studied, glucose concentration is lower in the fetal circulation than maternal circulation. Under normal physiological conditions, uterine glucose uptake is much higher than the net flux of glucose from the placenta to the fetus due to a large utilization rate of glucose by the placenta. Towards the end of gestation, fetal glucose requirements increase, as does the capacity for glucose transfer via an increase in GLUT 1 transporters. Wallace et al. (2003) demonstrated that placental size is the major limitation to glucose transfer in sheep.

Physiological and metabolic adaptions by the mother to allow for increased fetal glucose demand include a decrease in maternal glucose utilization and the use of alternative fuels such as free fatty acids, glycerol or glucogenic amino acids. Lactate is produced from glucose by the placenta under aerobic conditions and is transferred to the fetus by carrier-mediated mechanisms. The contribution of lactate to fetal metabolism amounts to one third or one half in most species but is minimal in the human (see Père, 2003 and references therein). Fructose is also produced by the placenta from glucose and may be involved in the synthesis of nucleic acids.

Protein is transported to the fetus in the form of amino acids by energy-dependent, stereospecific, amino acid transproter proteins. The plasma level of most of the amino acids is 2- to 3- fold higher in the fetus than the dam. The transfer from the mother of the essential amino acids is indispensable, but all the amino acids are not transported in a similar manner by the placenta. The placenta selectively takes up, metabolizes and transports each amino acid uniquely and it is beyond the scope of this chapter to detail all mechanisms. However, Hay (1994) reviewed in detail the placental transport mechanisms resulting from protein and amino acid metabolism.

All fetuses are able to synthesize lipids, but the great variation in fat content of the newborn (highest in humans and lowest in pigs) suggests differences between species in the rate of placental transfer of fatty acids or their synthesis by the fetus. In all mammalian species, non-essential fatty acids can be synthesized *de novo*, whereas essential fatty acids must be transferred from the mother. Placental lipid transfer involves direct transporter-mediated transfer of certain fatty acids, as well as lipid uptake from lipoproteins, metabolic alteration in the placenta and release into the fetal plasma. Triglyceride transfer has been shown in guinea pigs and rats (as reviewed by Garnica and Chan, 1996; Père, 2003) and VLDL-triglycerides are hydrolysed by an intracellular placental lipoprotein lipase before transfer to the fetus. Free fatty acids transferred across the placenta to the fetal circulation are esterified to triglyceride in the fetal liver.

2.1.4 Endocrine regulation of placental and prenatal growth

As well as the site of exchange between mother and fetus, the placenta is also a highly active endocrine organ, responsible for the production of a number of steroid, protein and polypeptide hormones, growth factors and other bioactive products.

Hormones and growth factors play important regulatory roles throughout pregnancy, modifying both maternal metabolism and uterine environment for the benefit of the conceptus. From implantation, through maternal recognition and maintenance of pregnancy, to parturition, each stage of gestation is dependent on a complex interaction of substances within the maternal-fetal placental unit. Several excellent reviews exist which summarize information on the various factors involved in the endocrine regulation of fetal and placental growth (Owens, 1991; Fowden, 1995; Garnica and Chan, 1996).

In all species, endocrine regulation of pregnancy initially involves hormones of the pituitary, ovary and placenta. Hormones have an important role in the control of fetal growth as they act on both tissue accretion and differentiation, and enable a precise and orderly pattern of fetal growth to occur during gestation. Substances produced by the maternal-fetal placental unit will be the main focus of this section, concentrating on the actions of the placental hormones and growth factors affecting placental and fetal development. Growth of the fetus and placenta represents one of the most extreme examples of rapid growth and development of normal tissue. The interactions between mother, placenta and fetus are complex and essential aspects of gestation, and therefore fetal and placental growth must be considered in an integrated manner.

2.1.4.1 Steroid hormones

In the pig, the corpus luteum (CL) remains the primary source of progesterone throughout pregnancy. However, the production and secretion of oestrogens and progesterone by the placenta are still essential for the maintenance and development of pregnancy. Oestrogens have been shown to increase uterine blood flow in the pig (Ford and Stice, 1985) and may have a role in fetal organ development and maturation. Oestrogen may also affect events leading to parturition.

Progesterone is essential for the maintenance of pregnancy. High local levels of the hormone block maternal cellular immune responses to foreign antigens like the fetus. Amongst its numerous roles, progesterone causes the development of the endometrium for the support and nutrition of the early embryo, decreases uterine contractility, modulates blood flow, and finally inhibits prostaglandin synthesis, which is critical in parturition (as reviewed by Garnica and Chan, 1996). Ashworth (1991) and Pharazyn et al. (1991) examined the relationship between plasma progesterone concentrations and embryo survival during early pregnancy in the pig. Concentrations at day 3 of pregnancy, and embryo survival to day 28 were positively related, suggesting that the timing of the initial increase in plasma progesterone may have a critical influence on embryo survival. A number of other studies support the concept that an early rise in plasma progesterone is supportive of good embryonic survival (see Foxcroft, 1997).

The corpora lutea of the pig also produce relaxin, which is a potent inhibitor of uterine contractions. Relaxin levels within the corpora lutea remain low during early pregnancy but increase dramatically as gestation progresses (Sherwood et al., 1975). During late pregnancy, relaxin acts as a cervical dilator and an inhibitor of myometrial activity (Sherwood, 1988). Relaxin gene expression has also been identified in the uterine endometrium during early pregnancy in the pig (Knox et al., 1994). This finding may implicate relaxin in stimulating uterine growth and function during placentation. Min et al. (1997) identified specific relaxin-binding cells in the porcine vagina and uterus using immunohistochemical localization techniques. In this study, endogenous relaxin was found to promote the growth of the vagina and the uterus in pregnant gilts. Min et al. (1997) also suggested that relaxin may assist the growth of the fetus by promoting growth and/or remodelling of the uterus during pregnancy.

2.1.4.2 Peptide hormones

Most placental protein and polypeptide hormones are structurally and functionally very similar to protein and polypeptide hormones produced by the pituitary and hypothalamus. For example, placental lactogens (PL) and chorionic gonadotrophins (CG) show similarities with prolactin (PRL) and luteinizing hormone (LH), respectively.

2.1.4.2.1 Chorionic gonadotrophins

Muyan and Boime (1997) reviewed the role of chorionic gonadotrophins in human pregnancy. In primates, the first embryonic signal necessary for implantation is the secretion of chorionic gonadotrophin (CG) by the trophoblast. hCG is detectable in the maternal circulation 8-10 days after ovulation, coinciding with blastocyst attachment and trophoblast formation. The levels rise exponentially, reaching a peak at 9-10 weeks of pregnancy and then decline steadily to term (Ogren and Talamantes, 1988). The rise in hCG is thought to be responsible for the rescue of the corpus luteum during early pregnancy and is essential for maintaining luteal function until the luteal-placental shift occurs. hCG overides the local luteolytic influence of the ovarian hormones and prolongs the life of the CL. Kuehl et al. (1992) measured chorionic gonadotrophin levels in pregnant baboons (bCG). They stated that the detection of static (rather than increasing) levels of bCG in early pregnancy can predict the occurrence of an impending spontaneous abortion. Studies reviewed by Muyan and Boime (1997) where active or passive immunoneutralization of CG results in early pregnancy loss, further support the critical role of CG in pregnancy maintenance.

As discussed previously in section 2.1.2.3, a different method of maternal recognition of pregnancy occurs in pigs. In the non-pregnant female $PGF_2\alpha$ produced by the uterus is released primarily into the uterine venous drainage for transport to the CL where it induces luteolysis to ensure that oestrous cycles are recurring until pregnancy is established. Bazer et al. (1986) described the antiluteolytic effects of oestrogen produced by conceptuses at the time of blastocyst elongation. Oestrogens act locally by causing a redirection of the secretion of luteolytic $PGF_2\alpha$ by the uterus. As a result of conceptus oestrogen production, $PGF_2\alpha$ is redirected into the uterine lumen where it is sequestered. Geisert et al. (1990) reviewed the possible luteotrophic action of oestrogen on the CL. It appears that oestrogen is capable of stimulating progesterone secretion by the CL possibly by an increase in LH, prolactin receptors and stimulation by catecholoamines.

2.1.4.2.2 Placental lactogens and Leptin

Placental lactogens (PL) are solely secreted by the placenta and are members of the growth hormone (GH)/prolactin (PRL) family. The production of placental lactogen reflects the development of the placenta, as blood PL levels rise throughout pregnancy and reach a peak at term (Walker et al., 1991). Placental lactogens and their receptors have been identified and purified from many species including the human (Hill et al., 1989), sheep (Warren et al., 1990a, 1990b), cow (Murthy et al., 1982) and goat (Currie et al., 1990). Several species have been reported *not* to produce a PL. These include the pig, cat, dog, and horse amongst others (Ogren and Talamantes, 1988) and other regulators of fetal and placental development must fulfill the role of the somatotrophic hormones seen in other mammals.

Symonds et al. (1998) reviewed evidence for a significant impact of maternal leptin status on feto-placental development. Leptin is a hormone secreted predominantly by white adipose tissue that is proposed to act on the hypothalamus to regulate food intake and the size of the body fat depots. However, a high abundance of leptin mRNA has been shown in the human placenta, with the highest amounts found in the chorionic villi at 8 weeks of gestation. It is speculated that leptin could have a similar role to that proposed for placental lactogen in promoting some of the adaptations of maternal metabolism during pregnancy (as reviewed by Symonds et al., 1998). Although the mechanisms mediating effects of leptin on fetal and placental development remain to be established, the concept of leptin as a regulator of feto-placental development is particularly interesting in the pig, which lacks a classic placental lactogen. Indeed, Ashworth et al. (2000) reviewed possible functions of placental leptin in a range of species, and suggested mechanisms of action including a role as a fetal and placental growth factor, promotion of angiogenesis, growth and maturation of the fetal immune system. In the pig, preliminary data suggested that placental leptin may be more abundant in placentae supplying normally grown fetuses than in placental tissue of IUGR fetuses from the same litter (Ashworth et al., 2000).

2.1.4.2.3 Insulin, Cortisol and Thyroid hormone

Insulin has long been recognized as a factor influencing fetal growth and plays a major role in promoting tissue accretion by increasing the mitotic drive and nutrient availability for tissue accretion. Insulin deficiency leads to severe IUGR as a result of defective substrate uptake (Gluckman, 1997).

Fowden (1995) reviewed work done in her laboratory involving the experimental manipulation of insulin levels which demonstrated that fetal growth rate in the sheep was directly related to insulin concentrations *in utero*. Fetal insulin deficiency was induced experimentally by surgical ablation of the fetal pancreas and by administration of diabetogenic drugs. Both procedures reduced fetal bodyweight near term by between 10 and 40% depending on the species and gestational age at onset of fetal hypoinsulinaemia. Insulin promoted fetal growth through its anabolic actions on fetal metabolism, increasing fat deposition and the uptake, utilization and oxidation of glucose by fetal tissues. Insulin may also act on growth by altering the concentrations of other growth promoting factors such as the insulin like growth factors (IGFs), which regulate cell division and differentiation, as discussed later.

The main effects of cortisol *in utero* are on tissue differentiation and maturation. Cortisol appears to act directly on the cells to alter gene transcription or post-translational processing of the gene products (Fowden, 1995). Thyroxine also plays a role in fetal growth by affecting both tissue accretion and differentiation.

2.1.4.2.4 Placental growth hormone

Pituitary growth hormone is a necessity for normal postnatal growth but appears to have little part in the control of prenatal growth (as reviewed by Fowden, 1995; Harvey and Hull, 1997; Harvey et al., 1998). Studies of decapitated/pituitary ablated embryos support the possibility that embryonic growth is pituitary GH independent. Campion et al. (1981) studied the effect of *in utero* decapitation of the fetal pig on skeletal muscle development and found that muscle and satellite cell ultrastructure were not altered by decapitation, leading to the conclusion that an intact brain and hypothalamic-hypophyseal axis are not critical. Similar experiments have been carried out in chick embryos, which were also found to exhibit normal growth following decapitation (as reviewed by Harvey et al., 1998).

However, it is now known that GH can be produced and act in many extra-pituitary tissues, raising the possibility that early embryonic growth may be dependent on the paracrine actions of extra-pituitary GH (Harvey et al., 1998). GH receptor (GHR) mRNA has been detected in fetal skeletal muscle by 75 days of gestation in the pig, which in contrast to the Campion et al. (1981) study, suggests that GH may play a role in fetal muscle growth (Schnoebelen-Combes et al., 1996). It is possible that fetal GHR may respond to the production and action of GH and GH-releasing factors outside the hypothalamic-hypophyseal axis.

One of the extra-pituitary sources of growth hormone appears to be the placenta. Frankenne et al. (1988) purified and partially characterised human placental growth hormone (PGH) from term placental extracts. Alsat et al. (1998) also studied the physiological role of PGH and concluded that it is the product of the GH-V gene specifically expressed in the syncytiotrophoblast layer of the human placenta. The human placenta secretes PGH in a non-pulsatile manner, from 15-20 weeks until term. PGH appears to have important implications for physiological adjustment to gestation, especially in the control of maternal IGF-I levels, as maternal plasma IGF-I correlates with plasma hPGH, irrespective of time of gestation. Both Frankenne et al. (1988) and Alsat et al. (1998) found that PGH appears to be selectively secreted into the maternal circulation, is undetectable in the fetal circulation and thus does not appear to have a direct effect on fetal growth. Specific GH receptors have been detected in the human placenta (as reviewed by Alsat et al., 1998). Furthermore, the presence of an immunoreactive GH releasing factor (GRF) has been detected in porcine placental extracts (Farmer and Gaudreau, 1997). Both findings suggest that the physiological role of PGH may include a direct influence on placental development via an autocrine or paracrine mechanism (Alsat et al., 1998).

2.1.4.3 Growth Factors

Various growth factors have been shown to play a role in the regulation of growth of the fetus and the placenta. These factors include insulin-like growth factors (IGFs), Epidermal Growth Factor (EGF), Transforming Growth Factor-β (TGF-β), Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (bFGF) amongst others. Brigstock et al. (1989) reviewed the roles of several of these factors. Although increasing numbers of growth factors and their receptors are being found in the placenta this section will briefly summarize information on a limited number of growth factors, which have been most extensively studied to date. These bioactive molecules are expressed in the female reproductive tract and in the fetus, and will collectively regulate the complex process of fetal and placental development.

2.1.4.3.1 IGF system

Two excellent reviews, which provide a good background to the insulin-like growth factors, their binding proteins and receptors, are by Jones and Clemmons (1995) and Hwa et al. (1999). As mentioned in the previous sections, one action of PL may be to modulate fetal IGF production, although this would not apply in the pig. The insulin-like growth factors (IGF)-I and -II are low molecular weight (approximately 7 kDa) single chain polypeptides, which are structurally similar to insulin (as reviewed by Hwa et al., 1999). The IGFs promote cellular mitosis and differentiation in a diverse variety of cell types and have been particularly implicated in fetal and placental growth in ruminants (Wathes et al., 1998), rodents (Van Kleffens et al., 1998) and in the pig (Lee et al., 1991, 1993; Persson and Rodriguez-Martinez, 1997).

The IGFs mediate their effects by binding to specific IGF receptors (type 1 and 2) on the surfaces of their target cells and circulate bound to a family of binding proteins. Six IGFBPs have been characterized and have been designated IGFBP-1 through to IGFBP-6. At the cellular level, IGFBPs are thought to inhibit the actions of the IGFs, but under specific circumstances, they may potentiate their metabolic and mitogenic effects (Jones and Clemmons, 1995).

The effects of IGFs on fetal and placental growth have been studied using transgenic and gene knockout mice. The advantage of these models is that the expression of a single gene can be changed via overexpression or deletion and then the effects on the whole organism can be studied (Van Kleffens et al., 1998).

2.1.4.3.1.1 Role of IGF in fetal growth

Both IGF-I and IGF-II and the type 1 IGF receptor are important for prenatal growth. IGF-II knockout mice showed a reduction in prenatal growth and a birthweight of 60% of normal. Ablation of the IGF-I gene also resulted in a similar reduction of birthweight and caused neonatal lethality in most mice. Null mutants for the type 1 receptor were even more reduced in birthweight (45% of normal) and all mice died after birth. These results suggest that the type 1 receptor serves as a receptor for both IGF-I and IGF-II in fetal development (Van Kleffens et al., 1998).

Kind et al. (1995) used a sheep model to demonstrate that restriction of placental size via endometrial carunclectomy, decreased the supply of oxygen and nutrients to the fetus, retarded fetal growth and reduced the concentration of IGF-I in fetal blood at 121 days of gestation (term = 150). In addition, the abundance of IGF-I mRNA in skeletal muscle, kidney and lung was reduced. In contrast, IGF-II protein in blood and IGF-II mRNA in fetal tissues was not altered by restriction of placental size. The authors suggested that altered production of IGF-I may contribute to retarded fetal growth, either through altered local actions or as a consequence of reduced circulating levels and thus through endocrine mechanisms.

Several papers provide evidence for the presence of IGFs and their receptors in the pig conceptus, suggesting a role for these factors in regulating the growth and development of the early blastocyst and fetus (Letcher et al., 1989; Corps et al., 1990; Chastant et al., 1994; Louveau et al., 1996; Gerrard et al., 1998). Lee et al. (1991, 1993) characterized the ontogeny of circulating IGFs and IGFBPs during fetal and early postnatal development in the pig. Serum IGF-II levels were shown to exceed IGF-I levels at all developmental stages and both factors exhibited postnatal increases. These results suggest IGF-II is a fetal and postnatal growth factor in the pig, while IGF-I is primarily a postnatal growth mediator.

2.1.4.3.1.2 Role of IGF in placental growth

IGF-II has been implicated in placental development in a number of species including rodents, primates and horses. Compelling evidence that the IGF system is implicated as one of the growth factor systems contributing to normal placental development, has come from gene deletion studies in mice. In mice with an IGF-II gene deletion, placental growth is arrested. In contrast, IGF-I deletion did not influence placental weight. Therefore it is unlikely that locally-produced IGF is important in murine placental development (as reviewed by Wathes et al., 1998).

Additional evidence for the importance of IGF-II in placental growth come from Coulter and Han (1996) who used immunohistochemistry to demonstrate that IGF-II is abundantly present in the rhesus monkey placenta and fetal membranes, whereas IGF-I is not detected at any stage. IGFR-1 mRNA was expressed predominantly in the decidua, as were IGFBPs 1-6 in variable abundance. These findings suggest a role for IGF-II in the regulation of nutrient transport or placental hormone synthesis and a role for IGF-II and IGFBPs in the cell to cell communication and interaction at the feto-maternal interface in the primate. Lennard et al. (1995) studied the expression of IGF-II mRNA in the placenta of the horse and concluded that IGF-II promotes invasiveness of the trophoblast and may have a possible role in the development of the placenta in the horse.

IGF-I mRNA expression has been found in porcine endometrial and conceptus tissue on days 8 to 30 of gestation (Letcher et al., 1989) and in sections of the endometrium and placenta of gilts up to day 40 of gestation (Persson and Rodriguez-Martinez, 1997). Simmen et al. (1992) investigated the temporal patterns of endometrial expression for mRNAs encoding IGF-I, IGF-II, IGFBP-2 and IGFR-1 in cyclic and pregnant pigs. Peak levels of IGF-I mRNAs were found at day 12 of cyclic and early pregnant gilts and decreased thereafter. Pregnant gilt endometrium had higher levels of both IGF-I and II mRNA than the corresponding stage in the cyclic gilt. IGF-II and

IGFR-1 mRNAs were low at day 12 and then increased with stage of pregnancy. Relative IGF-II mRNA tissue abundance was found to be placenta > endometrium > myometrium. These results suggested preferential roles for IGF-I at pre-implantation and IGF-II at post-implantation stages. They suggest that in the pig, IGF-I may function to regulate endometrial remodeling during the estrous cycle and implantation, while IGF-II is likely to mediate growth and differentiation of the endometrium and placenta during fetal development. The possibility was put forward that endometrium-expressed IGFBPs may play a major part in modulating the actions of the IGFs at the feto-maternal interface and within the individual tissue compartments.

Maternal nutrition during pregnancy influences fetal and placental weights. The effects of nutrition on fetal growth could be mediated by the IGF system in the uterus and placenta. Although local production of IGF by both the uterus and the embryo is important, the uterus will also be exposed to maternal circulating IGF.

2.1.4.3.2 Epidermal Growth Factor (EGF)

A good review of the metabolism and effects of EGF has been presented by Fisher and Lakshmanan (1990). EGF is a 53-residue polypeptide containing three disulphide bonds. It is synthesized as a large precursor molecule from which EGF and other EGF-like polypeptides (including TGFα, heparin binding EGF-like growth factor and amphiregulin are cleaved. The EGF superfamily exert their effects by interacting with the EGF receptor (EGF-R). The predominant biological effect of EGF on target cells is the enhancement of cell proliferation and differentiation.

Most studies on the role of EGF have been carried out in rodents. Fisher and Lakshmanan (1990) reviewed some of the roles of EGF which included precocious eyelid opening and tooth eruption in neonatal rodents, effects on fetal lung, gastrointestinal, liver and pancreatic maturation, adrenal and thyroid gland growth, mammary development, stimulation of wound healing and stimulation of CG and PL secretion from placental tissue. However the role of EGF in pregnancy and fetal development will be the main focus of this section.

Several studies have revealed the presence of EGF and EGF-related ligands in the female reproductive tract. Corps et al. (1990) identified the presence of EGF receptors on the preimplantation trophectoderm of the pig and Zhang et al. (1992) reported the presence of EGF receptors in the porcine endometrium during the first week of pregnancy and that EGF stimulated increased prostaglandin secretion. These studies suggest a role for a range of growth factors in the complex interactions between the uterus and the early blastocyst in the pig. Tamada et al. (1991) found TGFα mRNA expression in the luminal and glandular epithelium of the uterus during the peri-implantation period in the mouse, suggesting an involvement in preparation of the uterus for implantation and decidualization in rodents.

Vaughan et al. (1992) examined the expression of the genes for TGFα, EGF and EGFR during early pregnancy of the pig using RT-PCR. TGFα mRNA was expressed in the developing pig blastocyst at days 10,11, and 12 of pregnancy with peak expression coinciding with the onset of blastocyst elongation, which suggests a possible role for TGFα during this period of cellular remodeling. The temporal and spatial expression of EGF mRNA and protein was distinct from that of TGFα. EGF mRNA was first expressed by the post-elongation conceptus at around day 15 of pregnancy with levels increasing to day 22. EGF mRNA was found to be predominantly present in the embryo itself; the lung buds and gut loop of the day 22 embryos as well as the amnion. Endogenous EGF may therefore be associated with early organogenesis and with amniotic cell function.

Subsequently, Kennedy et al. (1994) detected mRNA for EGF, EGF-R, TGFα and Amphiregulin in both the porcine oviduct and the endometrium using RT-PCR and suggested that growth factors secreted into the lumen of the reproductive tract may supplement the embryonic production of these factors.

Peng et al. (1997) demonstrated that EGF and EGFR mRNA levels are developmentally and tissue-specifically regulated in the pig during later stages of pregnancy. The presence of EGF was detected in fetal pancreas, liver, kidney and skeletal muscle from day 90 of gestation. In the kidney and skeletal muscle, EGF mRNA increased with advancing gestation suggesting a role in muscle growth and maintenance during the later stages of fetal development.

Epidermal Growth factor and its receptor are also expressed in human placental tissue (Hock and Hollenberg, 1980; Bramley and Menzies, 1992) and EGF is presumed to play a role in feto-placental growth and development through its mitogenic action and by regulation of placental and fetal endocrine function. Lennard et al. (1998) also suggested a role for EGF in the growth of the allantochorion and endometrium during placentation in equids.

2.1.4.3.3 Transforming Growth Factor β (TGFβ) Family

Garnica and Chan (1996) reviewed some of the functions of TGFβ. The TGFβ family, which includes Activin and Inhibin, appears to be the most versatile of the growth factors. TGFβ is a multifunctional growth factor, which may act to either stimulate or inhibit cell differentiation. Hu et al. (1998) discuss one of the best characterized and most important functions of TGFβ which is its ability to arrest certain cells in the G_1 phase of the cell cycle. The deregulation of the ability of TGFβ to affect a G_1 arrest may contribute to oncogenesis.

TGFβ has been shown to have a role in embryogenesis, as well as the proliferation and differentiation of cells of connective, muscle, bone, cartilage, immune system, ovary, testis, adrenal, large and small blood vessels, liver and other tissues (as reviewed by Garnica and Chan, 1996). Hu et al. (1998) provided an excellent review of the molecular mechanisms by which TGFβ exerts its effects. TGFβ ligands signal by binding to specific receptors on the cell surface. Four receptors have been cloned although only two (Type I and Type II receptors) have been conclusively proven to mediate TGFβ signalling.

TGFβ mRNA expression was detected in the extra-embryonic trophectoderm and endoderm, in embryonic ectoderm and mesoderm, and also in the uterine luminal epithelium of the pig (Gupta et al., 1998a, 1998b). Again, these results suggest important roles for TGFβ in porcine conceptus development and in autocrine-paracrine interactions between the maternal uterus and the conceptus during early gestation.

Inhibin and activin are dimeric proteins which share sequence homology with TGFβ and thus belong to the TGFβ superfamily (Garnica and Chan, 1996). The family of inhibin-related proteins were first discovered in rete testis fluid (Setchell and Sirinathsinghji, 1972; Setchell and Jacks, 1974). Recently however, these proteins have been characterized as growth factors, embryo modulators and immune factors. Human placenta, amnion, chorion and maternal decidua express mRNAs for inhibin, activin and follistatin, and the presence of the bioactive proteins has been demonstrated (Petraglia, 1997).

Petraglia (1997) reviewed the structure and function of inhibin, activin and follistatin; Inhibin is composed of an α and β subunit. Two distinct β subunits are recognized and characterize two different forms of inhibin (inhibin A: αβA and inhibin B: αβB). Both forms of inhibin have an inhibitory effect on pituitary (FSH) release. Three forms of activin exist, distinguished by the combination of the two β subunits (activin A: $\beta_A\beta_A$, activin AB: $\beta_A\beta_B$ and activin B: $\beta_B\beta_B$). Activin acts to stimulate FSH release. Follistatin is chemically different and has no structural relationship with inhibin or activin. It is a single-chain glycoprotein, which inhibits FSH release by acting as a binding protein to neutralize the effects of activin.

Various biological actions have been described in embryonic and intrauterine tissues, which suggest a role for these proteins in the development of the feto-placental unit. One major action is the modulation of placental hormone secretion. Inhibin decreases, whereas activin increases, the release of hCG and progesterone from cultured placental cells (see Petraglia, 1997). So far there are no *in vivo* models to demonstrate the regulatory roles of inhibin-related proteins on placental hormone release. Rat, ovine and primate placenta has been found to express the various inhibin-related proteins but the biological roles of these proteins are likely to vary considerably between species due to the high species specificity of placentation and of endocrinology of gestation. Lockwood et al. (1997) analyzed serum levels of inhibin and activin in early human pregnancy. Elevated serum levels of inhibin A were detected in ongoing pregnancies from 4 weeks of gestation to an initial peak at 9-10 weeks of gestation, and significantly higher levels were found in multiple pregnancies compared to very low levels of inhibin A in non-viable clinical pregnancies. The study demonstrated that the elevation of circulating levels of dimeric inhibin A in early pregnancy is a result of production by the feto-placental unit. Furthermore, measurement of inhibin A may provide a rapid and early diagnosis of early pregnancy problems in clinical practice.

2.1.4.3.4 Platelet-Derived Growth Factor (PDGF)

Platelet-derived growth factor is a 30kDa platelet-contained serum mitogen that consists of two polypeptide chains (A and B). When combined as homo or heterodimers, the two polypeptide chains have different functional activities resulting from the different binding specificity of the two distinct classes of PDGF receptors (α and β) which are present on the surface of responsive cells (as reviewed by Persson and Rodriguez-Martinez, 1997).

Persson and Rodriguez-Martinez (1997) used immunocytochemistry to localize PDGF-A ligands and PDGF receptors during the establishment of the porcine placenta. The endometrial stroma, maternal epithelium, trophoblast and smooth muscle of the blood vessels on the embryonic side of the placenta showed immunolabelling. The authors suggest that these results indicate autocrine and paracrine roles for PDGF, as well as a role for this growth factor in the angiogenesis of the porcine placenta.

PDGF receptor α has been found to be expressed in the intra-placental yolk sac of the mouse placenta, indicating a role in murine placental development (Ogura et al., 1998). PDGF is also known to have a function in multiple processes of mouse development including angiogenesis, cardiovascular morphogenesis, lung branching, and retina development (Ogura et al., 1998 and references therein).

2.1.4.3.5 Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF)

The establishment of a vascular supply is a critical requirement for cellular inflow of nutrients, outflow of waste products and gas exchange in all organs including the placenta. Growth of the placenta requires extensive vasculogenesis and angiogenesis to develop its vascular structure in both fetal allanto-chorionic and maternal endometrium. The presence of known angiogenic growth factors such as bFGF and vascular endothelial growth factor (VEGF) in trophoblast cells has been reported using immunohistochemistry and *in situ* hybridization.

The molecular and biological properties of the VEGF family of proteins have been thoroughly reviewed by Ferrara et al. (1992). VEGF is an acid stable, dimeric, heparin-binding glycoprotein with a molecular weight of approximately 45 kDa. Cheung (1997) presented an excellent review of the role of VEGF in fetal development and placental function. Winther et al. (1999) used immunohistochemical methods to localize VEGF and its two specific receptors in the porcine placenta and non-pregnant uterus. They concluded that VEGF, Flt-1 and KDR ligand receptor system participates in the regulation of porcine placentation and that in addition to its angiogenic properties it may also influence the cellular differentiation and transport capabilities in uterine luminal and glandular epithelium and in the trophoblast. The two types of receptor for VEGF identified, designated KDR (kinase-insert domain-containing receptor) and Flt-1 (fms-like tyrosine kinase) belong to the family of receptor tyrosine kinases. Furthermore, both oestrogen and hypoxia induce VEGF expression and angiogenesis.

From a comparative viewpoint, VEGF is reported to mediate vascular changes and angiogenesis in the uterus during implantation and decidualization in the mouse (Halder et al., 2000). bFGF activity was examined in an experiment by Hamai et al. (1998) who demonstrated that cultured human trophoblastic cells obtained from early human placental villi release biologically active bFGF. In culture, angiogenic activity was eliminated almost completely by the concomitant addition of anti-bFGF antibody, suggesting that the angiogenic factor released by trophoblasts may be FGF. In the human, bFGF appears to be a crucial angiogenic factor derived from trophoblastic cells and thereby may play an important role in angiogenesis occurring early in gestation (Hamai et al., 1998). Failure to secrete the angiogenic growth factor may lead to failure in establishing the adequate placental vascular system and may cause pre-eclampsia.

2.2 Implications of placental function for fetal survival, development, the incidence of IUGR and occurrence of diseases in adulthood

With an understanding of the physiology and regulators of prenatal development, the second section of this review will focus on a range of environmental and genetic factors affecting placental and fetal development, which may have detrimental effects on pre- and postnatal growth. The consequences of inadequate or restricted growth *in utero* will be detailed. The phenomenon of fetal programming, whereby factors influencing the prenatal environment may initiate permanent changes in body structure and function, is of particular interest. Implications for postnatal growth performance in domesticated species raised for meat production and adult health in humans will then be considered. The effects of inadequate placental development on a wide range of body organ systems will be discussed in the context of the concept of brain-sparing effects.

2.2.1 Importance of adequate placental function

The adequate function of the placenta is essential for the growth of a normal fetus in all species. Failure of normal placental development is a frequent cause of intrauterine growth retardation or restriction (IUGR). Inadequate growth during intrauterine life has a detrimental effect on a diverse range of physiological functions in the offspring, many of which persist throughout life.

A significant relationship between placental and fetal weight at term is present in many species. Sanin et al. (2001) studied the relationship between birth weight and placental weight in 300 newborn babies in Mexico and found that for each gram increase in placental weight, birth weight increased by 1.98g. The relationship was non-linear (quadratic), due to placental aging after 40 weeks of gestation. In human pregnancies, the size and growth pattern of the placenta is an important predictor of birth weight and an indicator of disorders in fetal growth patterns. In the pig, fetal weight correlated well with placental area at all stages of gestation examined (day 38 until term; Wigmore and Stickland, 1985).

Various pathological conditions of the human placenta have been examined with wide ranging consequences, including postnatal skin conditions, thrombosis, low IQ, fetal malformations or death (Lewis and Gilbert-Barness, 1998). Placental insufficiency is also thought to be associated with an increased risk of permanent neurological disability, and the size of the placenta has been implicated as a component of the prenatal origins of hypertension (Barker et al., 1990) and cardiovascular disease (Osmond et al., 1993). Due to the significance of placental function in neonatal outcome, the evaluation of human placental pathology after parturition is strongly recommended, since the use of the placenta as a diagnostic tool may be helpful in the care of sick newborn infants. Although detailed postpartum evaluation of the placenta is generally not a concern in animal production, many researchers have examined the effects of experimental restriction of placental growth to determine possible causal factors for IUGR and consequences for postnatal outcome.

Experimentally-induced fetal growth retardation has been studied in a variety of animals; however, the majority of placental restriction studies have been carried out in sheep using a variety of technologies. Maternal nutritional restriction (Mellor, 1983), reduction of maternal uterine blood flow by either uterine or umbilical artery ligation (Emmanouilides et al., 1968) or embolization of the uterine or placental vasculature with microspheres (Creasy et al., 1972), limitation of placental capacity by removal of the endometrial caruncles before mating (Robinson et al., 1979) and maternal hyperthermia (Brown et al., 1977; Bell et al., 1987, 1989; Early et al., 1991) have all been used to reduce both placental and fetal growth and in extreme cases may lead to fetal death (as reviewed by Bassett, 1991; Hay, 1991; Robinson et al., 1994, 1995 and 2000; McMillen et al., 2001). Where relevant, the results of some of these studies and their effects on different aspects of fetal outcome are discussed in the following sections.

2.2.2 Factors affecting placental development and fetal growth

The growth of the placenta is influenced by many factors before and during early pregnancy. The initial conditions in the female reproductive tract, maternal body size, age, parity, genotype, nutrition, oxygen availability and the local metabolic, cytokine and hormonal environment of the embryo, all interact to affect the growth of the placenta (as reviewed by Robinson et al., 1997; Bauer et al., 1998a; Ashworth et al., 2001).

2.2.2.1. Initial conditions in the female reproductive tract

A continuing paradox of pregnancy is the survival of the embryo and placenta as an allograft. Presensitization of the female reproductive tract to paternal antigens before conception has been a subject of study in both humans and domestic species based on the assumption that immunological enhancement is an important phenomenon. Murray et al. (1983) used the gilt as a model to demonstrate that reproductive efficiency can be improved by intrauterine treatment with boar semen before a fertile insemination. Several studies have been conducted to examine the effect of the length of sexual cohabitation prior to human pregnancy (Clark, 1994). It has been observed that exposure of the reproductive tract of the woman to semen before conception is beneficial for the development of the placenta and the fetus. During a five-month study of 1011 consecutive deliveries, Robillard et al. (1994) found that the risk of developing pregnancy-induced hypertension in primiparous and multiparous women was inversely proportional to the length of sexual cohabitation. The risk of hypertension was significantly increased for women who conceived within the first 12 months of sexual cohabitation. Robillard and colleagues suggest that their observations are consistent with the hypothesis that during a protracted sexual relationship, women develop an immune response against spermatozoa which is not found in virgin women or in women using barrier contraceptives, and so should be immunologically protected against the father's antigens in a subsequent pregnancy. Clark, (1994) suggested that these findings support the hypothesis that repeated exposure to male ejaculate may also prevent pre-eclampsia.

Collectively, these data support the hypothesis that reproductive performance can be enhanced by the exposure of the uterus to sperm and/or seminal antigens before conception.

2.2.2.2 Maternal body size, age and parity

Maternal body size has a clear effect on fetal growth. Walton and Hammond (1938) conducted a series of crossbreeding experiments with Shire horses and Shetland ponies. The larger horse gave birth to a larger foal, irrespective of paternal genotype. Although the offspring of the crosses differed genetically from their respective mothers, at birth, the size of the fetus was approximate to that which the mother would normally give birth. Such effects of maternal body size have been seen in all species examined (Robinson et al., 1995).

In humans, small mothers produce smaller offspring than larger mothers, which may help to explain the tendency for the pattern of poor fetal growth in young adolescent mothers. The effects of maternal body size, age and parity on fetal development are likely to be interlinked and mediated through differences in uterine environment (hormonal and immunological), uterine capacity and nutrient partitioning between mother and offspring. Studies examining patterns of fetal and placental growth during adolescent pregnancy (Wallace et al., 1996, 2001, 2003) will be discussed in section 2.2.2.3. Constraint of fetal growth related to the intrauterine environment has been termed "maternal constraint" by Gluckman (1997). In pigs, it has been widely accepted that uterine capacity is the greatest constraint on litter size (as discussed in section 2.2.2.6); however, the efficiency of placental attachment and the ability of the placenta to supply the fetus with nutrients may also be critical determinants of fetal growth, as evidenced by studies in the prolific Meishan pig (see review of Ford, 1997).

2.2.2.3 Maternal nutrition

Nutrient supply to the fetus is a key factor in the regulation of fetal growth and has been the subject of many review papers including Godfrey and Barker (1995), Harding and Johnston (1995), Garnica and Chan (1996), O'Callaghan and Boland (1999) and Robinson et al. (1999). The appropriate growth and function of the placenta is essential for directing nutrients and oxygen to the developing fetus.

The effects of maternal undernutrition on fetal endocrine and metabolic status and on the interaction between the fetus, placenta and mother are complex and have been studied in a variety of species, including the human. Maternal undernutrition during gestation has been shown to commonly reduce both fetal and placental weight in experimental animals. However, the extent of reduction in weight depends largely on the severity and the timing of undernutrition and on the species studied (Harding and Johnston, 1995).

Of great interest are a series of studies conducted by Lumey (1992, 1998a, 1998b), which examined the effects of severe undernutrition at different stages of human pregnancy. Historical circumstances of the Dutch famine of 1944-45, which occurred as a result of an embargo on the transport of food supplies to western cities during German occupation of the Netherlands in World War II, provided an opportunity to analyse the relationships between intrauterine nutrition, birth weight and adult health, among a population relatively well defined by place and time. The famine was associated with a dramatic increase in mortality and morbidity. Pregnant women exposed to the famine were examined to study the effects of acute and short-term famine on infant birth weight and the subsequent reproductive performance of their female children. Intrauterine famine exposure was defined separately for trimesters 1, 2 and 3 as an average official ration of less than 4200 kJ daily in that trimester (less than 1000 kCal/day).

Results showed that exposure to the famine in late pregnancy (third trimester) reduced birth weight, whereas normal birth weight was observed if the fetuses were exposed to the famine in early pregnancy (first and second trimester). Second generation effects were also investigated by documenting the reproductive performance of female infants born before, during, and after the famine. Those who were of low birth weight due to maternal exposure to famine during late pregnancy gave birth to babies of normal birth weight. In contrast, although the fetuses that were exposed to famine *in utero* during early pregnancy grew normally, as adults those individuals gave birth to lighter babies. The reasons for the second-generation effect were unknown, but it was suggested that changes in the development of the uterus in fetal life might be responsible. Therefore, the prenatal timing of a nutritional insult rather than its effect on birth weight itself seems critical for any health effects on the next generation. This explanation fits with the theory of fetal programming, that different body tissues grow during differing "critical periods" of fetal development, as discussed in section 2.2.4.1.

A further analysis of the effect of undernutrition on human placental growth (Lumey, 1998b) found evidence for compensatory placental hypertrophy among infants exposed to famine in the first trimester of pregnancy, in that placental weight was increased at term, despite the fact that there was no change in birth weight in these individuals. In contrast, infants exposed to famine in the third trimester of pregnancy demonstrated decreased placental weights in addition to decreased birth weights (Table 2.1).

Table 2.1 Summary of the findings of the Dutch famine studies of Lumey (1998a, 1998b).

	Trimester of pregnancy		
Parameter	**1**	**2**	**3**
Birth weight	No effect	No effect	Decreased
Placental weight	Increased	Not stated	Decreased
2nd Generation birth weight effect	Decreased	Decreased	Normal

Lumey (1998b) interpreted the increase in placental weight in first trimester, famine-exposed, infants as compensatory response to a reduction in maternal caloric intake. However, it is not possible to know whether anatomical or functional changes in placental surface area or density might have occurred in conjunction with weight changes. Lumey (1998b) reviewed studies of maternal anaemia in which a similar increase in placental weight has been interpreted as compensatory placental hypertrophy for reduced oxygen supply to the fetus. Furthermore, similar patterns of placental growth have been observed in studies of placental adaptations in women living at altitude (as discussed in section 2.2.2.4). Additionally, the opposite association was reported by

Godfrey et al. (1996), in that increased caloric intake in early pregnancy, particularly associated with high carbohydrate and low protein intake, suppressed fetal and placental growth.

As discussed previously (section 2.2.1), in the majority of species there is a positive correlation between size at birth and placental weight, and generally fetal and placental growth is reduced by maternal undernutrition in experimental animals. Dwyer et al. (1992) found that growth of the guinea pig placenta was compromised by a 40% reduction in maternal feed intake, causing a 32% and 38% reduction in fetal and placental weights, respectively, with impaired or delayed expansion of the peripheral labyrinth in early gestation leading to reduced placental efficiency. Reduction in the growth of the feto-maternal exchange surface areas was suggested as a possible mechanism by which the effects of maternal undernutrition on fetal growth are mediated.

Studies on the effect of maternal undernutrition on placental and fetal development in sheep have revealed that effects on placental weight vary with time of nutrient restriction during gestation. Heasman et al. (1999) summarised results of a number of ovine studies and highlighted the inconsistency in effects on placental weight and suggested that factors such as initial maternal weight, body condition and breed may interact to confound results. Robinson et al. (1999) also reviewed a number of studies and concluded that the critical period of sensitivity occurs between approximately days 40 and 80 of gestation, which coincides with the period of rapid proliferative growth of the placenta. During this time the direction and magnitude of the placental response to nutrition is influenced by the size, body condition and degree of maturity of the ewe. For mature ewes, in good body condition at mating, a mild degree of maternal undernutrition from 30 to 90 days of gestation enhances placental growth, whereas for young ewes and those in poor condition, undernutrition has the opposite effect.

Further evidence for the effects of maternal nutrition and body condition on placental and fetal growth was provided in recent studies. Osgerby et al. (2003) investigated the effects of maternal body condition and nutrition on placental and fetal growth in mid-gestation ewes (gestation day 65). In this study, ewes with low body condition at mating exhibited greater mean placentome weight than animals with high body condition scores. As in the human studies discussed earlier, placental weight was affected, whilst fetal weight remained unaltered by maternal body condition and feed ration, suggesting that placental compensation is a very effective mechanism for maintaining fetal development over a considerable range of maternal conditions.

Conversely, as reviewed by Wallace et al. (2001), maternal overnutrition has been demonstrated to restrict placental growth and to perturb the normal development of the offspring. As briefly mentioned in section 2.2.2.2, human adolescent mothers are at increased risk of delivering low birth weight and premature infants. Wallace et al. (1996) developed a highly controlled model to investigate nutrient partitioning and the control of fetal growth in rapidly growing adolescent sheep. Rapid maternal growth rates from mating until day 95 of gestation were associated with a significant reduction in both fetal and placental weights and a reduced number of fetal cotyledons per placenta. The data indicate that in the adolescent female the hierarchy of nutrient partitioning is altered such that the anabolic drive to maternal tissue synthesis is maintained at the expense of the gradually evolving nutrient requirements of the gravid uterus. This overnourished pregnant sheep model replicates the adverse pregnancy outcome observed in human adolescents. Young, still-growing women appeared not to mobilize fat reserves late in pregnancy to enhance fetal growth, reserving them instead for their own continued development (Scholl et al., 1994).

Subsequent studies by Wallace and colleagues (as reviewed by Wallace et al., 2001) highlighted the importance of appropriate maternal nutrition during mid-pregnancy in setting the placental and hence fetal growth trajectory. Adolescent dams were switched from an anabolic to a catabolic state at day 50 of pregnancy and vice versa. A high plane of nutrition from day 50 was associated with a decrease in gestation duration, total placental mass and fetal cotyledon mass (the number of maternal caruncles having been determined by maternal dietary intake during the first 50 days of gestation). Reduced placental growth was associated with a major decrease in lamb birth weight at term, relative to treatment groups where a moderate quantity of diet was offered from day 50. Furthermore, it was demonstrated that nutrient transport function of the growth-restricted placenta could be altered in favour of fetal growth by induction of a catabolic phase at 100 days of gestation in previously rapidly growing adolescent sheep. The induction of a catabolic phase was associated with a sharp decrease in maternal insulin and glucose concentration, an increase in non-essential fatty acid concentrations, and an increase in mass of fetal cotyledons and fetal mass. The partitioning of nutrients and oxygen between the dam and the fetus is likely to be under the regulation of the complex endocrine systems discussed in the first section of this chapter.

Using the same adolescent sheep model, Da Silva et al. (2003) went on to examine the effects of maternal overnutrition during pregnancy on fetal body growth, pituitary gonadotrophin gene expression and gonadal development at day 103 of gestation. Their results showed that although overnutrition of the adolescent dam during the first two thirds of pregnancy had no effect on fetal weights, placental growth restriction was observed and fetal plasma progesterone concentrations decreased. Female, but not male, gonadal development was altered, as indicated by a decrease in the number of primordial follicles, and lower total follicle numbers in the ovaries of the female fetuses. This study provides supporting evidence to the results of the Dutch famine studies in the human, emphasizing that the development and function of the reproductive axis of the offspring is sensitive to maternal nutritional status during gestation, in addition to effects on fetal and placental weight.

As discussed by Robinson et al. (1997), poor nutritional status in the pig before establishing pregnancy is associated with decreased embryo survival, while a high level of nutrition increased the number of cells in the blastocysts. The mechanisms mediating nutritional effects on embryonic survival in pigs have been thoroughly reviewed by Foxcroft (1997). In part, effects of nutrition and metabolic state on embryonic survival are likely mediated by differences in maternal plasma progesterone, with high feed intakes in early gestation tending to decrease plasma progesterone concentrations due to increased metabolic clearance of steroids in these circumstances. Clearly, the effects of overnutrition on offspring development are different between single offspring and litter bearing species. Evidence for the detrimental effect of overnutrition of the adolescent dam on fetal development is lacking in the pig, although studies of the response to super-alimentation of first parity lactating sows suggested preferential partitioning of additional nutrients to maternal growth rather than to milk production (Pluske et al., 1998). The effects of maternal nutrition on prenatal growth of the pig and particularly development of the fetal muscle fibres will be discussed in detail in the third section of this chapter.

In summary, nutrition clearly has an important role in the regulation of fetal growth and effects vary with the timing and severity of the insult. Clearly nutritional effects are far more complex than simple deprivation of substrate supply. The sheep studies described, raise interesting questions regarding the interactive effects of maternal nutrition and the degree of maturity of the dam on uterine blood flow and placental growth, as well as the possible involvement of endocrine factors such as maternal insulin and the IGFs, which are likely to be involved in nutrient partitioning between mother and fetus. Furthermore, critical windows of sensitivity to maternal nutrient intake have been shown to exist. As outlined in the human studies of the Dutch Famine, sub-optimal maternal nutrition may lead to fetal adaptations that have little effect on birth weight *per se*, but may have long-term effects on structure and function. For this reason, birth weight is likely to be an inadequate measure of normal fetal development.

2.2.2.4 Supply of oxygen

In their extensive review of the literature, Kingdom and Kaufmann (1997) classified the origins of fetal hypoxia as preplacental, uteroplacental or postplacental. In preplacental hypoxia, both the placenta and the fetus become hypoxic because of reduced oxygen content within the maternal blood. Examples of this include pregnancy at high altitude or maternal anaemia. Uteroplacental hypoxia occurs when normally oxygenated blood has a restricted entry into the uteroplacental tissues due to occlusion or failed trophoblast invasion. Cases of uteroplacental hypoxia include pre-eclampsia (maternal hypertension). Postplacental hypoxia is characterised by a major defect in fetoplacental perfusion, which prevents the fetus from receiving sufficient oxygen, even if maternal blood enters the intervillous space at a normal rate. Examples of this type of hypoxia include certain types of IUGR and experimental umbilical cord ligation. Patterns of fetoplacental angiogenesis and villous growth vary in pregnancies complicated by different forms of fetal hypoxia.

Robinson et al. (1995) reviewed some of the evidence that the placenta alters its growth in response to modest oxygen deficiency. Interestingly, the placenta also shows significant adaptations in women living at altitude. Lower birth weight occurs at altitude, while placental weight remains unchanged or is increased. Enhancement of oxygen transfer may be facilitated by movement of the fetal capillaries to the periphery of the villi and can be shown quantitatively by increased oxygen diffusion conductance of the villous stroma. Mayhew (2003) and Tissot van Patot et al. (2003) described the remodelling of fetal capillaries and uteroplacental arteries in placentae from high altitude pregnancies. Mayhew (2003) highlighted some of the inconsistencies in the findings from high-altitude investigations on human and animal placentae and suggests possible explanations for discrepancies, including effects of confounders such as pre-adaptation to high-altitude environment, ethnicity or species, maternal anthropometry and maternal nutritional, iron, socio-economic and reproductive status. A more detailed review of the adaptations of the human placenta to the various types of placental hypoxia is not appropriate but the results of a few comparable studies of hypoxia conducted in animals are outlined in the following paragraphs.

In sheep, Owens et al. (1987) found that restriction of placental growth by endometrial carunclectomy, led to a reduced supply of oxygen to both the pregnant uterus and the fetus (preplacental hypoxia), and a redistribution of oxygen to the fetus. This was due to the disproportionate maintenance of fetal growth relative to that of the placenta, since oxygen consumption by either, in terms of tissue, mass was not altered.

Gagnon et al. (1995) determined the effect of daily fetal placental embolization on DNA synthesis rates in the ovine fetus and placenta. Fetal arterial oxygen content was decreased by 30 to 35% of pre-embolization values by the injection of latex microspheres into the fetal circulation. The microspheres obliterated the small arterioles on the fetal side of the sheep placenta. Intravenous injection of tritiated thymidine , andmeasurement of subsequent incorporation into DNA, was used to determine rate of DNA synthesis. Following 10 days of embolization, fetal organ weights, placental weight, placental/body weight ratio or fetal organ/body weight ratios were not significantly altered. However, reduced DNA synthesis was observed in trophoblast cells of cotyledons, quadriceps muscle and the left ventricular myocardium. It was concluded that the reductions in the DNA synthesis of these tissues are the earliest adaptive mechanisms of fetal growth to placental insufficiency.

Subsequently, Mallard et al. (1998) used the same method of microsphere injection into the umbilical circulation to impair placental function and examine the effects of induced chronic fetal hypoxemia on brain development in sheep. Placental insufficiency was induced from 120-140 days of gestation. Although there was no reduction in fetal brain weight in hypoxemic fetuses compared with controls (indicative of brain sparing; see section 2.2.3.2), various changes in neurodevelopmental processes were observed, including reduced myelination of cortical white matter and reduced growth of the cerebellum. It was concluded that placental insufficiency resulting in moderate fetal hypoxemia damaged the fetal sheep brain, and by affecting neural connectivity could have functional consequences after birth.

2.2.2.5 Effect of genotype

The Chinese Meishan pig is noted for its prolificacy, averaging three to five more pigs per litter than either American or European breeds. For this reason, it has been used in a range of experiments to investigate the effect of genotype on embryonic survival and size of the fetus and placenta. Reciprocal embryo transfer between Landrace X Large White gilts and Meishan gilts demonstrated clearly that maternal genotype has a major influence on fetal size (Ashworth et al., 1990). Day 30 fetuses carried by Meishan dams were lighter than fetuses carried by the Landrace X Large White recipients regardless of the genotype of the fetus. The impact of uterine type and conceptus genotype on development through late gestation in pigs has also been investigated in a series of experiments using embryo transfer (Youngs et al., 1994; Ford, 1997; Wilson et al., 1998; Biensen et al., 1998, 1999). Increased prolificacy of the Meishan was suggested to be due to an increased embryonic survival resulting from a suppressive effect of the uterus on embryonic growth rate and estrogen secretion (Youngs et al., 1994). Studies of placental and fetal development at later stages of gestation and to term (Biensen et al., 1998; Wilson et al., 1998) are in agreement with earlier data from Ashworth et al. (1990) and show that regardless of genotype, fetal and placental weights at day 90 were markedly smaller when recovered from Meishan than Yorkshire recipients. Data obtained from these reciprocal transfer experiments suggest that placental size is largely determined by the uterine environment, regardless of fetal genotype until approximately day 90. After day 90, fetal demand for nutrient uptake and waste removal increases rapidly. These demands seem to activate fetal breed-specific mechanisms to facilitate increased feto-maternal exchange. Yorkshire conceptuses respond by dramatically increasing placental size between days 90 - 110, with no change in placental vasculature. In contrast, Meishan conceptuses enhance feto-maternal exchange by nearly doubling the vascularity of their placentae between days 90 - 110 with no increase in placental surface area, resulting in an increased placental efficiency. The suggestion was put forward that after day 90, Meishan conceptuses may initiate increased synthesis and secretion of angiogenic factors to facilitate the increased vascularisation of the placenta. Biensen et al. (1999) found that fetal genotype-specific differences in placental and endometrial vascular density, previously seen for straightbred Meishan and Yorkshire conceptuses were eliminated by crossbreeding (Meishan X Yorkshire fetuses gestated in straightbred Meishan or Yorkshire gilts). Furthermore, the placental efficiency was intermediate for Meishan X Yorkshire conceptuses compared with straightbred Meishan or straightbred Yorkshire conceptuses, regardless of the uterine type in which they were gestated. Collectively, these data indicate that whilst uterine type determines conceptus size, conceptus genotype determines placental efficiency.

Vonnahme et al. (2002) investigated the effect of conceptus genotype on compensatory increases in placental size in response , the loss of an adjacent littermate. At day 40, alternate fetuses were destroyed by crushing in one uterine horn while the other horn was left as a control. No differences were observed in fetal weight or crown rump length between treated or control horns of females of either breed. However, placental weight, surface area and implantation site length were increased in treated horns versus control horns of Yorkshire females whilst no differences were observed in placental parameters between horns in the Meishan. These data indicate that differences exist in the strategies employed by Meishan and Yorkshire conceptuses in the competition for nutrients during gestation. Yorkshire conceptuses were seen to accelerate placental growth when adjacent littermates perished.

Genetic differences in placental size and vascularity may therefore be important factors affecting litter size in swine. As well as accounting for the greater potential for increased litter size in the Meishan pig compared with US and European breeds, variation in placental efficiency within breeds may also exist (Wilson et al., 1999), and may offer opportunities to increase litter size regardless of the inherent uterine capacity of the dam.

2.2.2.6 Littersize, uterine capacity and location in the uterus

The effect on embryo survival of the number of embryos *in uV2* and the amount of uterine space available has been a subject of extensive research for several decades. The concept of uterine capacity as a determinant of littersize has been researched by a number of authors using a variety of different methods. A number of animal models have been developed to study uterine crowding in the pig. Dziuk (1968) used several surgical methods to test for the effect of uterine crowding by changing the length of uterine segment available to each embryo through uterine ligation, oviduct resection, unilateral hysterectomy/ovariectomy, superovulation, and egg transfer technologies. Only when the average number of embryos was at least 14 did it appear that intrauterine crowding was a limiting factor for littersize born.

Bazer et al. (1969a, 1969b) further defined the concept of uterine capacity, concluding that increased death associated with increased embryo numbers was due to maternal limitations and not to inherent limitations of the embryo. A uterine mechanism is thought to limit litter size to a level characteristic for the species or breed and Bazer and colleagues suggested that the ability of the uterus to support embryonic development, involved two physiological mechanisms; initially, embryo selection may occur as the result of the more viable embryos being better able to compete for some biochemical factor in the uterus which is necessary for their continued development. Later in gestation, intrauterine competition for the establishment of adequate surface area for nutrient exchange between fetal and maternal circulation may act to reduce litter size. The same group of researchers (Fenton et al., 1970) went on to investigate the stage of gestation when uterine capacity b, a limiting factor for fetal survival and concluded that it was after 25 days of gestation. Studies of feral Ossabaw swine (Hagen and Kephart, 1980; Hagen et al., 1980, 1984) found similar effects of uterine crowding on embryonic survival and litter size and their results indicated that uterine capacity of feral swine is limiting after day 30 of gestation.

The initial length of the uterus appears to be an important limit to litter size as the number of ovulations (and therefore potential embryos) increases (Wu et al., 1987, 1988b, 1989). A longer uterus was associated with a greater number of live fetuses and a lower incidence of mummified fetuses, and 36 cm of uterus per fetus was determined as the requirement for implantation, survival and full development.

Extensive studies carried out at Clay Center, Nebraska have revealed that uterine capacity is a characteristic that can be selected for in breeding programs. Christenson et al. (1987) used the unilateral hysterectomy-ovariectomy (UHO) model to evaluate uterine capacity in swine. Compensatory ovarian hypertrophy occurs when one ovary is removed (Staigmiller et al., 1972), however, uterine compensation does not occur. It was concluded that the UHO model results in litter sizes that are an estimate of uterine capacity for one uterine horn, which is not confounded by ovulation rate. Vallet (2000) summarized experiments examining the effect of uterine crowding on litter size and fetal survival and concluded that in most cases conceptus survival in crowded uteri was high prior to day 30, but was impaired by day 35 to 40.

Gama and Johnson (1993) evaluated the results of eight generations of direct selection for litter size born and an increase in ovulation rate (1.30 ± 0.38 eggs) and uterine capacity (0.66 ± 1.28 pigs; measured after UHO) was achieved. Although no significant changes were found in uterine dimensions, the number of mummified pigs at birth was reduced, indicating that uterine capacity in late gestation had increased. The responses obtained in first parity gilts were maintained in second and third parity sows. Subsequently, Johnson et al. (1999) reviewed the results of selection for litter size over fourteen generations of swine. They concluded that genetic improvement programs should emphasize live born pigs because of undesirable genetic relationships of ovulation rate and number of fetuses with numbers of stillborn and mummified pigs, and because birthweight decreased as litter size increased.

Père et al. (1997) evaluated the effects of the number of pig embryos on fetal survival and growth, and maternal metabolism, in 114 Large White gilts. Their data supported the hypothesis that uterine capacity limits litter size in sows, and in contrast to the data of Dziuk (1968), they suggested that even sows with a normal ovulation rate are affected. An ovulation rate greater than the number of pigs the sow is able to keep alive until farrowing resulted in lighter fetuses at term and uterine space seemed to be involved in these effects. Although Père et al. (1997) reported that the number of conceptuses reflecting uterine capacity was usually determined by fetal mortality before 35 days of gestation, compensation between late and early fetal mortality was demonstrated in gilts with a normal to high embryo potential (control and UHO gilts versus oviduct ligated gilts). When early fetal mortality was high, late fetal mortality was low, and vice versa.

As well as affecting littersize born, uterine crowding has also been shown to have effects on fetal growth, probably due to small placental contact area (Knight et al., 1977). Differences in fetal weight based on position within the uterus have been observed in a number of studies (Waldorf, 1957; Perry and Rowell, 1969; Dzuik, 1985; Ashworth et al., 2001). Waldorf noted the negative correlation between number of fetuses and fetal weight as early as 1957 and examination of position within the uterus showed that fetuses and membranes at the extremes of a uterine horn were larger than those towards the middle.

These findings are in agreement with those of Ibsen (1928) in guinea pigs. Perry and Rowell (1969) also noted that the fetuses at the ends of the horns tended to have an advantage over those in the middle of the horns. Furthermore, they observed an increase in weight of fetuses at the ovarian end compared to the cervical end of the horn. Examination of the uterine vasculature provided no obvious relationship between the uterine vascular architecture and fetal weight. In a study of more than 400 pregnant pigs, Dziuk (1985) noted that the amount of uterine space available to each fetus decreased from the ovarian to the cervical end of the uterine horn. By day 40 of gestation, space was similar at the two ends of the uterine horns, however, those in the middle had the least space and were most likely to be smaller at birth than their littermates.

Wise et al. (1997) found no relationships between fetal weight and position on day 30, however, on days 70 and 104 of pregnancy, the heavier fetuses were found at the ovarian ends and the light fetuses at the cervical ends of the uterine horns. Data relating position within the uterine horn and fetal size are equivocal. Most descriptions of the relationship between uterine position and fetal size are confounded by littersize or by the number of fetuses in each uterine horn. Ashworth (1991) used a formula to calculate relative uterine position, which expressed the position of each fetus within the uterine lumen on a uniform scale from 0 (ovarian) to 1 (cervical). The lightest fetuses contained within the smallest placentae were located at the ovarian end of the uterine horn, with fetuses and placentae becoming heavier towards the body of the uterus. However, using the same formula, no evidence was found that a particular position in the uterine horn was associated with advantages for fetal growth (Ashworth et al., 2001).

Wise and Christensen (1992) examined the effect of fetal sex on placental and fetal development in the pig and noted that from day 70 of gestation, male pig fetuses and their placentae are heavier than female fetuses and placentae. At day 104 of pregnancy, sex of neighbouring fetuses within the uterus affected fetal size. Specifically, they reported that a fetus with both neighbours of the opposite sex was lighter in weight than a fetus surrounded by neighbours of the same sex. It was proposed that fetuses with neighbours of the opposite sex may represent litter runts that never develop "normally" postnatally or animals with later reproductive unsoundness. The authors suggested that fetal weight differences may be related to immunological differences between fetuses, or alternatively that endocrine influences of adjoining fetuses may have occurred early during development (day 20-40) but did not appear until the growth phase (day 75-110).

Khong et al. (2003) examined the increase in birth weight in second and subsequent born human children. Their results suggested that permanent anatomical changes in the spiral arteries of the uterus might modify subsequent vascular remodelling in the next pregnancy. In addition to explaining the increase in birth weight with parity, permanent anatomical changes in the uterus may be a mechanism of increased litter size with increasing parity in pigs. In contrast, a decrease in birth weight with successive pregnancies in humans was observed by Lumey (1998a), which may have been related to maternal nutritional status. However, results of birth weight/parity studies seem to be equivocal.

2.2.2.7 Environmental temperature

Damanhouri and Tayeb (1992) present a comprehensive review of the available methodology for a variety of heat stroke studies. The effect of environmental temperature on various measures of reproductive function has been the subject of a number of studies in experimental animals. The majority of studies have examined the maternal endocrine and fetal metabolic responses to heat stress exposure in sheep. Heat stress primarily reduces placental growth and the associated retardation of fetal growth represents a fetal adaptation to a decreased placental ability to supply oxygen and nutrients (Bell et al., 1987, 1989; Vatnick et al., 1991).

Dreiling et al. (1991) found that uterine blood flow decreased 20 to 30% in pregnant ewes subjected to a 1°C increase in body core temperature. It was concluded that heat stress stimulated the release of maternal antidiuretic hormone or oxytocin, which reduced uterine blood flow and caused a shift in fetal metabolism from anabolic to catabolic pathways. Chronically heat-stressed ewes produced lower body weight IUGR offspring which also displayed effects of brain sparing as evidenced by a decreased liver weight compared to brain weight (see section 2.2.3).

Galan et al. (1999) investigated whether fetal IUGR caused by heat stress beginning at day 35 of gestation was reversible on removal of the heat stress after 55 days versus 80 days. They found that the longer the exposure, the greater the effect of IUGR on the fetus and the placenta.

Pigs are particularly susceptible to large variations in environmental temperature, especially heat stress, since they do not sweat. Pregnant females during late gestation are also more susceptible to exhaustion and death after exposure to elevated ambient temperatures (Omtvedt et al., 1971). Wettemann and Bazer (1985) examined the influence of elevated environmental temperature on prolificacy of pigs. Studies suggested that heat stress during early pregnancy alters the reproductive endocrine system, especially the control of luteal function. Gilts were especially susceptible to heat stress during early pregnancy. Decreased conception rates and reduced litter sizes were observed when gilts were exposed to elevated ambient temperatures during days 0 to 16 after mating. Reduced conception was linked to reduced plasma progesterone and increased concentrations of estradiol during days 10 to 12 of heat stress were suggested to interfere with maternal recognition of pregnancy. Heat stress also reduced the amount of embryonic tissue present at day 16 of pregnancy. Omtvedt et al. (1971) examined heat stress of gilts during later gestation. Reproductive performance of gilts subjected to heat stress during mid pregnancy (53-61 days postbreeding) was not significantly different to control animals. Exposure of gilts to heat stress at 102-110 days of gestation however, decreased the number of live pigs from 10.4 ± 0.76 to 6.0 ± 0.76 and increased the number of dead pigs at farrowing from 0.4 ± 0.62 to 5.2 ± 0.62. It was concluded that gilts were most susceptible to heat stress during early and late pregnancy.

2.2.2.8 Other environmental factors

Various environmental teratogens or toxins have been linked with placental and fetal growth deficiency. Maternal ethanol ingestion is associated with a number of teratogenic effects, among them pre- and postnatal growth deficiency (Garnica and Chan, 1996). Another environmental factor shown to affect placental and fetal development in humans is maternal tobacco smoking. Smoking is associated with the occurrence of smaller placentae and a thickening of the villous membrane in early pregnancy, necrosis of the trophoblast and a reduction in the capillary volume of the villi in later pregnancy (as reviewed by Robinson et al., 1995). These factors presumably contribute to the reduced birthweight caused by maternal smoking.

2.2.3 Physiology and measurement of Intrauterine Growth Retardation

Intrauterine growth retardation or restriction (IUGR) is the failure of the fetus to grow properly *in utero*. Restricted fetal growth is a common occurrence in many species and is associated with increased perinatal morbidity and mortality. Whilst perturbations of fetal growth have been recognised and documented for centuries, more recently fetal growth restriction has been associated with a variety of adverse long-term health outcomes, which will be discussed in section 2.2.4. As previously discussed, experimental studies in animals have shown that some of these detrimental outcomes can readily be induced by restriction of placental and thereby fetal growth, enabling the study of consequences of specific fetal adaptations to a poor intrauterine environment.

From an obstetric viewpoint, the obvious importance of prenatal growth for fetal and neonatal well-being has meant that human intrauterine growth has received considerable attention. Graphs that depict normative data for birth weight, length and head circumference at various gestational ages have become traditional tools in perinatal medicine since the early 1960s. Several classic standard growth curves exist for humans (Lubchenco et al., 1966; Sparkes et al., 1998 (review)) typically spanning the last trimester of pregnancy and generally composed of static cross-sectional measurements as opposed to longitudinal studies. Despite their widespread use, there are numerous theoretical concerns and practical problems associated with intrauterine growth curves. For a concise review of the disadvantages of intrauterine growth curve use, see Sparkes et al. (1998). Briefly, theoretical concerns include the legitimacy of estimating a dynamic measurement of rate of change for the longitudinal growth of a hypothetical fetus, based on differences among static measurements of different fetuses following delivery. Furthermore the use of percentile data at a given gestational age to construct an intrauterine growth curve for an idealized fetus presumes that a single growth curve in fact exists. The interactions of genetic and environmental factors in determining birth weight and postnatal growth (as first observed by Walton and Hammond, 1938) clearly suggest that it may be more accurate to use a family of growth curves describing intrauterine growth in differing populations and thereby select the best curve based on knowledge of the individual fetus.

Practical problems in the analysis of intrauterine growth include accurate estimation of gestational age, and inaccuracy of physiological measurements during or after delivery, which may be distorted by intrapartum events and may not be representative of the same measurements *in utero*. Ultrasound monitoring during pregnancy has the advantage of allowing serial measurements for an individual subject, however, measurement error may be large relative to fetal size. Obviously longitudinal ultrasound studies are easier in the human or in monotocous animal species, whereas the ability to measure the same fetus *in utero* sequentially in a polytocous species such as the pig is limited.

Clinical nomenclature differs to describe variations in fetal growth and many terms are in common usage including *low birth weight* (LBW), *very low birth weight* (VLBW) and *extremely low birth weight* (ELBW), which refer to human infants with birth weights less than 2500g, 1500g and 1000g, respectively. However, these terms do not incorporate the concept of gestational age which is described by a different set of terms; small for gestational age (SGA) refers to individuals below the 10th percentile adjusted for gestational age, whilst large for gestational age (LGA) refers to infants above the 90th percentile adjusted for gestational age. Those between the 10th and 90th percentile are appropriate for gestational age (AGA). The terms IUGR, SGA and FGR (fetal growth restriction) seem to be used interchangeably by clinicians and researchers with the same statistical limits used to define each (i.e. individuals below the 10th percentile adjusted for gestational age). This has led to debate regarding the appropriate boundaries used to classify growth in fetuses and infants. Mamelle et al. (2001) attempted to distinguish between SGA infants that should be considered "constitutionally small" since they failed to meet a standard weight (or length) threshold for their gestational age, and FGR infants referring to individuals that failed to achieve their genetic growth potential *in utero* and could therefore be considered as growth-restricted. They concluded by calling for a universal definition of FGR based on the collection of a very large population of data from both developed and developing countries including maternal, sociodemographic and anthropometric factors. However, despite studies such as these, to date there seems to be continuing overlap and discrepancies between terminology and statistical limits used to define intrauterine growth.

The physiology and pathology of retardation of human fetal and placental growth have been described in a number of studies (Gluckman and Harding, 1997; Salafia, 1997; Sparkes et al., 1998). Two major patterns of fetal growth have been observed in human fetuses exhibiting diminished fetal growth, both of which may predispose individuals to a range of disorders in adult life (as discussed in section 2.2.4). The first growth pattern is that of symmetrical IUGR where the fetus grows at a constant but slower rate than normal. This is typical of a limited growth potential that may be hereditary or congenital – an intrinsic failure of fetal growth. The second pattern of growth is asymmetrical, where the rate of growth slows and may even stop. Brain growth is relatively preserved, whereas growth of the liver, spleen and somatic tissues are affected resulting in disproportionate body measurements, a phenomenon described as *brain sparing*. Typically no single causative factor can be clinically identified in cases of asymmetrical growth retardation, although it is thought to be secondary to effects on placental function. Any of the factors discussed in section 2.2.2 are likely to be involved, particularly decreased maternal supply of nutrients or oxygen to the placenta or decreased placental substrate transfer.

As noted by Sparks et al. (1998) there are more data describing growth of humans than of experimental animals. However, studies of the human IUGR neonate are limited by ethical considerations, and whilst care must be taken when extrapolating results of animal studies to humans due to substantial biological diversity, clearly the study of intrauterine growth of other species provides insight into understanding human data. A variety of animal models have been used for the study of IUGR, and in most cases involve surgical intervention. Moreover, naturally occurring IUGR has been documented in a wide variety of species, including the pig, which has long been suggested to be an appropriate model for the human infant (Glauser, 1966; Cooper, 1975). Similarities between the newborn pig and the human neonate include a comparable level of maturity at birth, some anatomical similarities, susceptibility to hypothermia, shivering thermogenesis ability and increase in metabolic rate in the first few days of life. Obvious differences exist in terms of differing birth weight and subsequent weight gains, inability of the pig to sweat, contrasting body temperature and body fat, as well as the obvious contrast of a singleton birth versus a litter bearing species. Despite these differences, one advantage of the use of a litter-bearing species is that siblings can be used as experimental animals and controls.

2.2.3.1 Nature and timing of IUGR in the pig

In pigs, as in other species, fetal growth retardation is associated with reduced birth weight and increased risk of fetal and neonatal death. The study of intrauterine growth in the pig is not a new area of research and studies go back many decades (Lowrey, 1911; Warwick, 1928; Waldorf et al., 1957). Whilst of interest, the relevance of this information to current studies is questionable since there is no doubt that the prolific dam-lines in commercial use today are very different from the animals examined nearly a century ago.

A limited amount of data on the relationship between fetal development and gestational age exists in the pig in the form of intrauterine growth curves, similar to those widely used in human perinatal medicine. Pomeroy (1960) plotted the average weights of 'normal' fetuses (excluding 'obviously abnormal or degenerated fetuses') with stage of pregnancy (51, 74, 97, 106, 108 and 110 days of gestation) and found a cubic relationship (Figure 2.4). A total of 80 sows of various breeds and parities were used, 70 of which were at known stages of gestation, with gestational age of the remaining 10 being estimated with reference to the fetal growth curve derived from the sows of known stages.

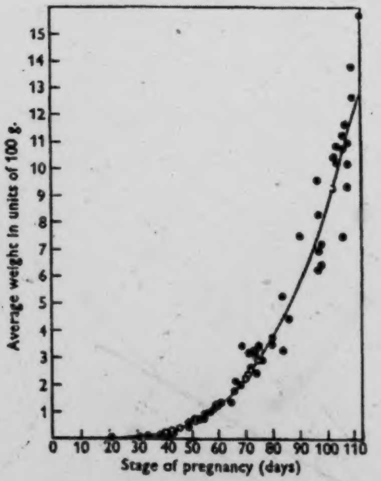


Figure 2.4 Cubic relationship between average weights of 'normal' fetuses with stage of pregnancy (51, 74, 97, 106, 108 and 110 days of gestation (from Pomeroy, 1960).

Marrable and Ashdown (1967) published figures describing the relationship between both embryonic length and embryonic weight and gestational age using 241 pig embryos and fetuses from 26 litters ranging in age from 26 to 109 days of gestation (See Figure 2.5). Whilst embryo length was shown to have a linear relationship with gestational age, embryo weight was best described by a polynomial fit (4th degree). Huge variance was observed around the mean embryonic weight and length values during later gestation (approximately day 80 onwards). The reliability of the data of the Marrable and Ashdown study was questioned by Cooper and John (1977) who suggested that the variance of the data was likely caused by the method of measurement which they found to be unreliable in fetuses over 60 days of age, and was especially disparate in fetuses over day 90 of gestation. A comparison of fetal weights at day 90 of gestation using the curves of Pomeroy (1960) and Marrable and Ashdown (1967) give values of 600g and 650g, respectively.

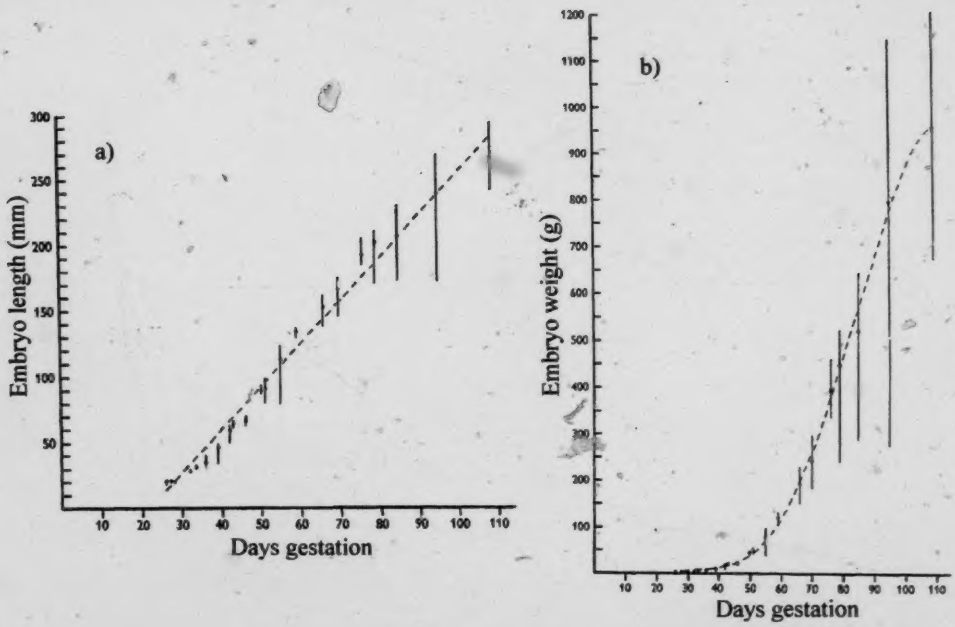


Figure 2.5 The relationship between a) Embryonic length and b) Embryonic weight and gestational age for 241 pig embryos. The range of weights and lengths are shown by continuous vertical lines and the respective means by a dot. Regressions are shown by a broken line (adapted from Marrable and Ashdown, 1967).

Most recently, Flecknell et al. (1980) measured body and organ weights in piglets from 44 litters delivered by caesarean section at various times during the last 20 days of gestation, in an attempt to assess the usefulness of these measurements as predictors of gestational age. Whilst the authors found a highly significant correlation between mean litter weight and age, they conceded that a considerable degree of uncertainty about the true gestational age must be accepted if mean litter weight is to be used as the sole predictor, since they found the 95% confidence limits to be ± 10.2 days at a mean litter weight of 1 kg. The authors also concluded that no extra information was afforded by the inclusion of fetal organ weight data for predicting gestational age, once the mean litter weight was known. Furthermore, these authors were critical of previous research involving calculations of standard growth curves in the pig, mainly due to the fact that they have been based on relatively few observations.

Considering the problems surrounding the use of growth curve data, criticisms of measurement technique (Cooper and John, 1977), and the relatively limited number of litters used for analysis (with no consideration of litter size effects on fetal development or weight), fetal growth curve data in the pig should be interpreted with caution. Whilst growth curves may be useful to estimate approximate gestational age in cases where time of mating is unknown, the usefulness of existing porcine fetal growth curves for assessing the developmental age of suspected IUGR fetuses is likely to be highly inaccurate. A definitive classification of the limits determining IUGR would still be required for data falling below the defined growth curves.

Many definitions have been used to describe inadequately grown fetuses in a litter (as briefly discussed by Ashworth et al., 2001) and the classification of "runt" is a somewhat arbitrary term (Powell and Aberle, 1980). Early studies in the pig have used descriptions of the characteristic appearance of the smallest individual of each litter (lean conformation and domed head; Cooper, 1975; Cooper et al., 1978), arbitrary weight group classifications (Powell and Aberle, 1980, 1981; Linderkamp et al., 1981), those individuals weighing only one half or even one third as much as the largest littermate (Widdowson, 1971; Adams 1971; Dickerson et al., 1971; Flecknell et al., 1981a, 1981b) and individuals less than two-thirds of the mean weight of the other fetuses in the uterine horn or largest littermate (Perry and Rowell, 1969; Hegarty and Allen, 1978). Animals shown to be outliers in an otherwise normally distributed population have also been classified as IUGR individuals (Cooper et al., 1978; Royston et al., 1982; Flecknell et al., 1983; Wootton et al., 1983; van der Lende et al., 1990; Xu et al., 1994; Da Silva-Buttkus et al., 2003). Growth-restricted animals have also been chosen based on position within the uterus, with fetuses in the middle of the horn classified as those most likely to be growth-restricted (Waldorf et al., 1957; Perry and Rowell, 1969). More recently, in experimental studies using ovine models of restricted placental growth, IUGR has generally been classified as a fetal or neonatal body weight below two standard deviations of the expected weight for gestational age, or more frequently of the mean of the relevant study population (McMillen et al., 2001). As in human studies, IUGR pig fetuses have also been classified as weighing less than the 10th percentile (Bauer et al., 1998b, 2002).

A number of studies have examined the time point in gestation when IUGR offspring may first be identified. In comparisons of the largest and smallest fetuses within a litter, Pomeroy (1960) described significant differences in litters at 74 days but not at 54 days of gestation. Cooper et al. (1978) detected runting (offspring with body weights two standard deviations or more below the mean litter weight) at day 44 of gestation. Subsequent studies have reported that within-litter variation in piglet birth weight appears to be established by the end of the first month of pregnancy. Perry and Rowell (1969) reported finding runts (defined as fetuses less than two thirds of the average for the horn) in litters of gestational age 31 to 49 days. Knight et al. (1977) characterised conceptus (placental membranes, fetal fluids and fetus) development in intact control (IC) and unilaterally hysterectomized-ovariectomized (UHOX) gilts between days 20 to 100 of gestation to evaluate the effect of intra-uterine crowding in UHOX gilts having essentially the same number of potential embryos but only one half the endometrial surface area of IC gilts. They found no differences in fetal survival, crown-rump length or fetal weight prior to day 35, however, placental mass was significantly greater in IC gilts at all stages of gestation, indicating that IUGR effects on placental weight are detected prior to effects on fetal weight. Van der Lende et al. (1990) also concluded that variation in within-litter weights was established early in gestation, prior to the end of the embryonic stage of development (day 35).

2.2.3.2 Consequences of IUGR in the pig

In addition to classification criteria for IUGR pig fetuses and neonates, several researchers have studied the pathological features of IUGR in greater detail and a plethora of evidence is available regarding the detrimental effects of IUGR on pre and postnatal development in the pig. Widdowson (1971), Cooper (1975), Flecknell et al. (1981a) and Bauer et al. (1998b) examined the effects of IUGR on body weight and organ size. A common finding of these studies was that the brain was least affected by growth retardation in comparison to other organs. Widdowson (1971) found that the growth of the liver was retarded more and the growth of the brain less than that of the body as a whole. It was demonstrated that the organs and muscle of the 'runt' pigs were smaller than those of their large littermates and contained less protein and DNA. Furthermore, the brain, heart, spleen and stomach of the full-term runts were larger than those of normal fetuses of the same weight; however, muscles were smaller and contained less DNA than the organs of normal fetuses. Dickerson et al. (1971) found that the brains of IUGR piglets weighed less and were less highly developed in terms of cell number, cell size, degree of myelination and dendritic development than those of the large littermates, indicating that the brain is not completely "spared" during IUGR; however, they did not compare relative brain weights (i.e. brain to body weight ratio) or the ratio of brain weight to other organ weights such as the liver. Cooper (1975) noted the value of the ratio of organ weights as a useful measure of growth retardation. As a result of the visceral organs being affected to a greater extent than the central nervous system in cases of IUGR, the brain:liver ratio of growth-restricted animals is greater than that of 'normal' individuals. Cooper cited the example of 2 piglets, both full term weighing 1216g and 339g; the brain/liver ratio of the former being 1.0/1.7 (= 0.58) but of the latter 2.5/1.0 (2.5). Similarly, Flecknell et al. (1981a) found that liver, kidneys, heart, lungs, spleen and pancreas all showed a significant positive relationship with bodyweight. In contrast, brain, pituitary, adrenal and thyroid weights did not change significantly with bodyweight, implying preferential protection from the pathological effect of intrauterine growth retardation. The more recent study by Bauer et al. (1998b) found a significantly increased brain to liver ratio in animals with a body weight below the 10th percentile, providing further evidence for the brain sparing effect. They concluded that extreme body weight variation is most probably caused by alterations of placental function resulting in asymmetrical growth retardation.

The effects of growth restriction have been observed on many aspects of development. Adams (1971) concluded that growth-restricted fetuses had smaller bones, which were anatomically and chemically less mature than those of well-nourished fetuses of the same age. Deroth and Downie (1978) found negative effects on haematological and cardiovascular parameters of underweight neonatal swine versus normal fetuses of the same weight.

Flecknell et al. (1981b, 1983) examined the rate of total body glucose turnover and cerebral blood flow and metabolism in normal and IUGR piglets. Cerebral blood flow was 35% lower in IUGR versus normal fetuses but the rates of cerebral utilization of oxygen and glucose were not significantly different between the two groups. The normal utilization rates were achieved by an increase from 50 to 70% in IUGR piglets in the fractional extraction rate of arterial oxygen by the brain. These findings have important implications for increased susceptibility to the development of hypoxic brain damage should changes in oxygen supply occur. In addition, increased glucose utilization rates in IUGR fetuses could place high demands on the gluconeogenic capacity of the liver and rapidly deplete glycogen reserves in their relatively undersized liver, leading to the development of hypoglycaemia. The piglet is a particularly appropriate model for the study of glucose homeostasis in the human infant since both the human and porcine neonate tends spontaneously to develop hypoglycaemia.

Xu et al. (1994) examined the effects of IUGR on gastrointestinal morphology and enzymatic maturity in newborn pigs. They found that weight and cell number of the pancreas and gastrointestinal tract were decreased in cases of IUGR. The GI tract was proportionally small compared to the body as a whole, while the pancreas was disproportionately smaller. Bauer et al. (2002) reported detrimental effects on renal function in newborn IUGR piglets, which exhibited a reduced glomerular filtration rate and osmotic clearance. The number of kidney nephrons was markedly reduced in neonatal IUGR piglets however, the number of nephrons was related to body weight, so it was unclear whether reduced renal function was purely a characteristic of decreased body weight rather than a true effect of growth retardation.

The reproductive axis has also been shown to be affected by IUGR. Da Silva-Buttkuss et al. (2003) found that IUGR affected ovarian organogenesis. Ovarian mass was reduced in association with the reduction in body weight. However, the ovaries of

IUGR neonates had a reduced number of primary follicles and an absence of secondary follicles. Runt piglets had more primordial follicles indicative of delayed follicular development. Germ cell proliferation and differentiation were not seen to be affected, nor was follicular cell degeneration (measured by TUNEL-assay). An important consideration is whether ovarian changes initiated during fetal life are maintained during postnatal life and will impact on adult reproductive performance. IUGR has also been shown to detrimentally affect porcine skeletal muscle development and this will be discussed in section 2.3.6.

As discussed in this section, many different definitions of IUGR have been used in studies of restricted uterine growth in the pig and detrimental effects on the development of numerous organ systems have been observed. Whilst statistical definitions of IUGR such as a fetal or neonatal body weight below two standard deviations of the mean of the study population are required to detect statistical differences between experimental groups of animals, it is likely that in reality the effects of IUGR are more complex and may exist as a gradient of effects of growth restriction. Also, effects of IUGR on fetal growth are generally accepted to be secondary to changes in placental function. Therefore, it is likely that detrimental effects on placental development will be the earliest sign of IUGR (as seen by Knight et al, 1977). Likewise, perturbations in body organ development may occur before an overall effect on body weight is observed. Clearly, thresholds for IUGR on various developmental parameters need to be identified in the pig and these are likely to be influenced by a combination of genotype, parity and maternal health status.

2.2.4 Placental and fetal growth and long term health outcome

The "fetal origins of adult disease" hypothesis is also known as the "Barker Hypothesis", since it was David Barker who first noted that coronary heart disease may originate from impaired development *in utero* and during infancy (Barker et al., 1989). Barker's early studies of the relationship between size at birth and mortality due to adult ischaemic heart disease, led to the hypothesis that the intrauterine environment and particularly fetal undernutrition at different stages of gestation can be linked to patterns of early growth and in turn this exerts important long-term effects on susceptibility to adult diseases. The role of prenatal programming has become increasingly clear and has led to suggestions for modifications and additions to the original hypothesis. In particular, studies of the Dutch famine during World War II discussed in section 2.2.2.3, led Lumey (1998a) to suggest that the fetal origins hypothesis should be modified to recognise the fact that "long term health effects after fetal undernutrition may occur in the absence of a birth weight effect, and may not be apparent even in its presence".

Controlled animal-based studies provide insights into the molecular, cellular and systemic mechanisms that contribute to the different manifestations of fetal programming, a number of which have been presented in previous sections of this chapter. In the following sections, the concept of fetal programming will be discussed further and evidence for the role of early developmental growth patterns leading to specific disease outcomes will be presented from epidemiological studies.

2.2.4.1 Fetal programming

The phenomenon of fetal programming, whereby undernutrition in early life resulting from inadequate maternal intake of food or inadequate transfer of nutrients permanently changes body structure and function is well documented in animals (as discussed in sections 2.2.2 and 2.2.3 of this chapter). During fetal growth and development, the various tissues of the body grow during different 'critical periods' of rapid cell division. The concept of fetal programming is that a stimulus or insult during these sensitive periods of early development (rapidly growing tissues are more vulnerable) may alter expression of the fetal genome and have permanent effects on the body's structure, physiology and metabolism. The processes that underlie the changes in structure and function, include reduction in cell numbers, changes in the distribution of cell types and in organ structure, including vascularisation and resetting of hormonal feedback.

A number of different systems and body tissues may be affected by fetal programming, including the cardiovascular, respiratory, endocrine, and immune systems, and skeletal muscle, bone, liver and kidney (as reviewed by Godfrey, 1998). Experimental studies in animals have documented many examples of fetal programming. Alterations in maternal diet during gestation have been shown to program permanent changes in the offspring, including changes in blood pressure, cholesterol metabolism, insulin secretion, as well as tissue-specific effects resulting in changes in muscle fibre number (Dwyer et al., 1995; Godfrey, 1998 and references therein). De Bruin et al. (1998) examined the effects of fetal growth retardation on human ovarian development. IUGR fetuses had significantly lower volumes of ovarian primordial follicles compared to age-matched control fetuses. Findings that ovarian development is impaired by IUGR suggest that as a result of premature loss of follicles, females with low birth weight may encounter fertility problems in later life. The critical periods during gestation that affect fetal muscle fibre development will be discussed in section 2.3.

The associations between neonatal size and adult diseases are thought to result from "fetal programming" during gestation. As discussed in section 2.2.3, two major patterns of fetal growth have been observed in human fetuses exhibiting diminished fetal growth. It is thought that exposure to an adverse intrauterine environment at different stages of gestation has effects on body proportions of the human neonate. Follow-up studies have shown that this predisposes them to different disorders in adult life.

As discussed by Godfrey and Barker (1995) and Godfrey (1998), proportionately small babies are at increased risk of raised blood pressure but do not appear to develop coronary heart disease (CHD), while disproportionately small babies tend to have abnormalities of systems controlled by the liver, including cholesterol metabolism and clotting factor synthesis, and have increased rates of CHD. It has been suggested that *in utero*, these babies may have invoked the brain-sparing reflex later in gestation, thereby diverting available nutrients to the brain at the expense of the trunk, limbs and abdominal viscera including the liver. In addition to CHD, babies who are disproportionately small at birth, or who have altered placental growth, have an increased incidence of hypertension, non-insulin-dependent diabetes and cardiovascular disease in adult life (Barker et al., 1990, 1993a; Godfrey and Barker, 1995; Godfrey, 1998). Babies that are thin at birth are at increased risk of insulin resistance syndrome (Thompson et al., 1997) and CHD in later life. Thinness at birth is thought to reflect reduced subcutaneous fat and skeletal muscle as a consequence of fetal undernutrition in the weeks prior to delivery.

Conflicting evidence exists in support of the hypothesis that proportionate and disproportionate growth retardation is associated with temporal differences in intrauterine growth. A study carried out by Vik et al. (1997) assessed intrauterine growth by prenatal ultrasonography measurements and found no difference in growth at different times in gestation between fetuses with the two different body proportions. They also found no evidence of brain sparing in disproportionately small babies.

The predominant phenotype of fetal growth restriction and the mix of babies born with different body proportions vary in different populations. This suggests that the long-term patterns of fetal growth restriction may contribute to geographical variations in the prevalence of CHD (Godfrey, 1998).

2.2.4.2 Epidemiological studies linking fetal and placental development with diseases in adulthood

The hypothesis that the intrauterine environment may exert important long-term effects on susceptibility to CHD originated from studies of death rates among babies born in Britain during the early 1900s (Barker and Osmond, 1986). At this time, the usual certified cause of death in newborn babies was low birth weight. Neonatal death rates differed considerably throughout the country. It was observed that the geographical pattern in death rates closely resembles today's large variations in death rates from CHD. One conclusion drawn from this observation was that low prenatal growth rates are linked to the occurrence of the disease in later life.

Further, more direct evidence of *in utero* programming of the disease came from studies of men and women born in Hertfordshire, UK between 1911 and 1930 in which size at birth was related to the occurrence of CHD in middle age (Barker et al., 1989;

Osmond et al., 1993). Death rates from CHD were seen to fall with increasing birth weight in both sexes (Godfrey, 1998).

Barker et al. (1993b) linked reduced rates of fetal growth to raised serum cholesterol concentrations in adult life. The authors suggest that impaired liver growth may permanently alter low density lipoprotein cholesterol metabolism. Consistent with these findings, babies with retarded fetal growth have been shown to be at increased risk of developing increased blood pressure and elevated plasma concentrations of glucose, insulin, and fibrinogen in addition to LDL cholesterol - all of which are risk factors for cardiovascular disease (CVD) during adulthood. The associations between size at birth and CVD are independent of adult weight, social class and smoking, and have been consistently found in different populations (Godfrey, 1998).

2.2.4.3 Genetic imprinting

Much of the early epidemiological and animal research on fetal programming focused on the effects of poor nutrition during pregnancy. While factors such as vascular determinants of placental uptake and transfer of nutrients have been extensively investigated over the past decades, especially in sheep and rodent models, genetic factors have been somewhat ignored until much more recently. Young (2001) and Reik et al. (2003) have written excellent reviews on the connection between imprinted genes and the Barker hypothesis, and the role of imprinted genes in the regulation of supply and demand for maternal nutrients in mammals.

The expression of imprinted genes depends on their parental origin. Whilst most genes are expressed from both parental loci simultaneously, imprinted genes are only expressed from either the maternal or the paternal allele. Reik et al. (2003) estimated that approximately 100 imprinted genes are likely to be found in mammalian genomes, however, many of the imprinted genes so far identified are involved in the control of fetal growth and are expressed in both fetal and placental tissues, making them plausible candidates for fetal programming.

The evolution of imprinted genes is explained by the genetic conflict hypothesis, whereby paternally-expressed imprinted genes enhance fetal growth, whilst maternally-expressed genes act to suppress fetal growth. Paternally-derived genes are selected to extract more resources from the mother, to provide more nutrients to the fetus, whereas maternally-derived genes are selected to conserve resources and maximize reproductive performance of the female over successive pregnancies. The provision of nutrients to the current fetus must be balanced with the nutrient requirements for future fetuses from the same mother (but potentially different fathers). Since imprinting is thought to be absent in fishes, reptiles and amphibians, which are generally egg-laying, Reik et al. (2003) suggested this provides further support for the genetic conflict hypothesis.

The characteristics and wide variety of mechanisms regulating imprinted gene expression may act at both the genetic (gene sequence) and epigenetic (modifications which alter DNA structure and function rather than sequence) levels. Genetic features include repeat DNA sequences near imprinting control regions and antisense promotor sequences. Epigenetic DNA modifications include DNA methylation, histone acetylation and differences in chromatin structure. A detailed summary of these mechanisms is beyond the scope of this chapter, however, readers are directed to an excellent review by Reik and Walter (2001). Imprinting is thought to be established during gametogenesis but precise timing of establishment is still unknown (see review by Young, 2001).

A number of reviews have examined the role of imprinted genes in determining fetal and placental growth (Moore, 2001; Young, 2001; Miozzo and Simoni, 2002; Reik et al., 2003). Recent studies on *Igf2* (insulin-like growth factor 2) knockout mouse models provide experimental evidence for the hypothesis that imprinted genes have central roles in controlling both the fetal demand for and placental supply of, maternal nutrients. Mouse knockout studies also provide additional evidence for the genetic conflict theory. Paternally-imprinted genes act to increase fetal size and, in keeping with this hypothesis, knockouts of the paternally-expressed genes *Igf2*, *Peg1* and *Peg3* resulted in deficits in placental size (as discussed by Reik et al., 2003). Maternally-imprinted genes act to decrease fetal size; therefore, knockout mice for maternally-expressed *H19*, insulin-like growth factor 2 receptor (*Igf2r*) and *p57Kip2* all resulted in hyperplasia of the placenta.

Reik et al. (2003) also suggested that imprinted genes might affect fetal nutrient supply by affecting placental transport mechanisms including transporters and channels involved in transplacental solute exchange. Examples of these genes included *Slc22a2* and *Slc22a3* genes, which are linked to *Igf2r* and are maternally expressed. The *Ata3* gene, which is paternally expressed, encodes a component of the system A amino acid transport system of the placenta. *Ata 3*, in addition to *Ata 1* and *2* which neighbour it, are of interest since the system A is upregulated in the *Igf2* knockout mouse (see Reik et al., 2003 and references therein). Reik et al. (2003) also suggested that imprinted genes could regulate nutrient supply and thereby affect placental function by affecting overall growth of the whole placenta, or by affecting particular placental compartments (such as the labyrinthine trophoblast), alternatively, specific placental transport systems may be affected.

Both Young (2001) and Reik et al. (2003) proposed that nutrition plays a role in imprinting. Young (2001) postulated that environmental factors, including nutrition, might directly affect the imprinted loci. There may be indirect effects on expression, action or accessibility of DNA methyltransferases that regulate or maintain imprints, or indirect effects on availability of transcription factors that maintain normal allelic expression. At least in terms of DNA methylation, there is now a growing body of evidence supporting the link between nutrition and epigenetic modification.

Collectively, the recent data from the mouse studies reviewed by Reik et al. (2003), led the authors to suggest that the "changes in expression of imprinted genes could provide a genetic link between the provision of nutrients, the intrauterine growth rate, and the programming of fetal systems determining the risk of disease in adulthood". However, nutritional effects need to be studied specifically at the imprinted loci to investigate the interactions of nutrition, gene imprinting and pre- and postnatal health and development.

2.2.5 Summary of factors affecting programming of adult disease

Fetal development is regulated by a number of factors including fetal genome, maternal body composition, dietary intake and metabolic state, utero-placental blood flow and placental transfer. When nutrient demand exceeds materno-placental supply, changes in fetal metabolism and endocrine function occur which may result in changes in fetal development which impact on later life, and especially a predisposition to diseases of the cardiovascular system (see Figure 2.6). The varying critical periods during which organs and systems mature indicate that an adverse intrauterine environment at different developmental stages is likely to have specific short and long term developmental effects on the different organ systems of the offspring. Programming is now known to be an important underlying feature of many systemic adult diseases including coronary heart disease, hypertension and insulin resistance syndromes.

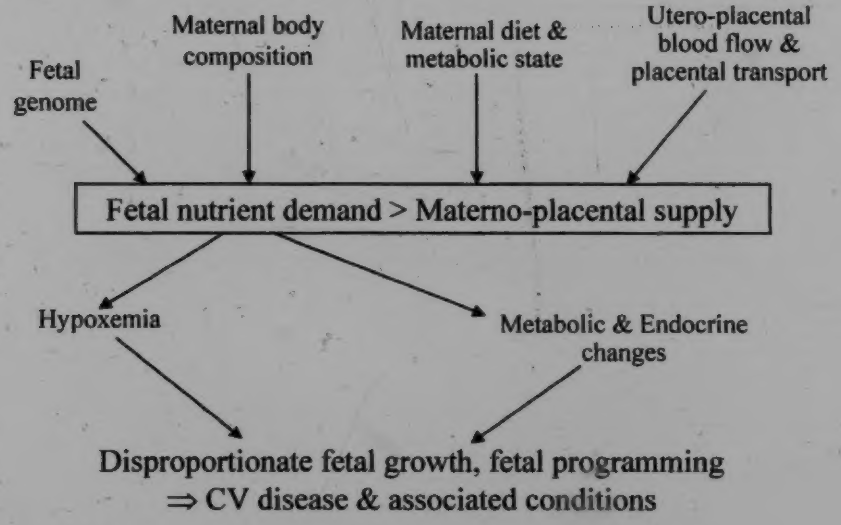

Fetal genome
Maternal body composition
Maternal diet & metabolic state
Utero-placental blood flow & placental transport
Fetal nutrient demand > Materno-placental supply
Hypoxemia
Metabolic & Endocrine changes
Disproportionate fetal growth, fetal programming
⇒ CV disease & associated conditions

Figure 2.6 Summary of factors affecting programming of adult diseases.

Adult cardiovascular disease may be a consequence of fetal adaptations invoked when the materno-placental supply fails to meet fetal demand. Epidemiological studies have led to the recognition that variations in fetal size and thinness at birth have implications throughout life.

The various effects observed following experimental placental restriction (decreased DNA synthesis, effects on the IGF and the sympathoadrenal system, decreased availability of oxygen) are likely to be the type of factors that have implications in later life. They may contribute to the increased incidence of various disease conditions in the adult human. Although long term consequences of fetal exposure to various restrictive conditions *in utero* are still not completely understood, increasing evidence suggests that the uterine environment may initiate morphologic and/or functional changes within the fetal organ systems that are subsequently amplified to impact health and the incidence of disease in postnatal life. Research in this area needs to progress beyond just looking at epidemiological associations, to a greater understanding of the molecular and cellular processes that underlie them. The phenotype of offspring subjected to a variety of intrauterine challenges is determined by lifelong gene expression patterns set into motion during critical windows of development. In some instances these phenotypes pass on their effects to future generations.

Imprinted genes may provide a system for nutrient-gene interactions that modify the balance between the supply and demand of nutrients *in utero* with major implications for fetal growth and the intrauterine programming of adult disease.

2.3 Importance of prenatal events for postnatal muscle growth in pigs

The final section of this review will examine how the range of environmental and genetic factors discussed in the previous section affect skeletal muscle development. The principles of muscle structure and function are described, with a focus on factors that regulate myogenesis. The importance of muscle development in terms of postnatal growth and meat quality in the pig are discussed.

2.3.1 Functional importance of skeletal muscle

Skeletal muscle is essential for a wide range of functions including breathing, thermogenesis, maintenance of posture and locomotion. Defects in normal muscle development could impair any of these functions in the neonate and this has important implications for the survival and well being of all mammals. In the pig, skeletal muscle plays an essential part in maintaining homeothermic balance and hence neonatal survival. Since the newborn pig does not possess brown adipose tissue, it relies almost exclusively on shivering thermogenesis for thermoregulation (as reviewed by Herpin et al., 2002)

The major components of a given muscle are the muscle fibres, the formation of which are completed late in gestation in mammals and at hatching in avian species. Muscle fibre differentiation is determined by prenatal events and the number of muscle fibres and the growth rate of the individual muscle fibres determine the postnatal growth rate of muscles. Muscle growth is of particular interest in agricultural animals because of its obvious commercial importance. Goals of animal breeding include the production of animals with a greater mass of muscle and faster rate of muscle growth.

2.3.2 Basic principles of muscle development in the pig

The biphasic nature of muscle fibre formation has been well established in the pig and the critical periods of fetal muscle development during gestation have been identified (Wigmore and Stickland, 1983 and references therein). In early embryonic development mesenchymal cells differentiate into committed myogenic precursor cells. These precursor cells are mononucleated myoblasts. According to the biphasic theory, an initial population of primary fibres develops first between 35 and 55 days of gestation, by the rapid fusion of myoblasts to form primary myotubes. Myotubes are elongated multinucleate cells, which do not divide. Later in gestation, between 55 and 90 to 95 days, a second generation of myotubes appears and forms the main bulk of the muscle. The secondary fibres form around the primary myotubes, using them as a framework, which guides their growth towards the tendons. The total number of fibres is considered to be definitively established by 90-95 days gestation although hypertrophy and muscle maturation continues. However, hyperplastic growth may not cease at birth, as previously thought, and a tertiary generation of muscle fibres may be formed postnatally as discussed in section 2.3.7.1. Figure 2.7 summarises the biphasic nature of myogenesis in the pig.

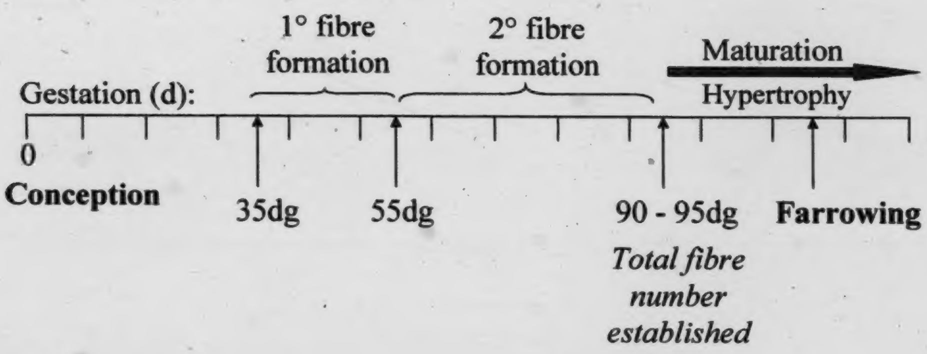


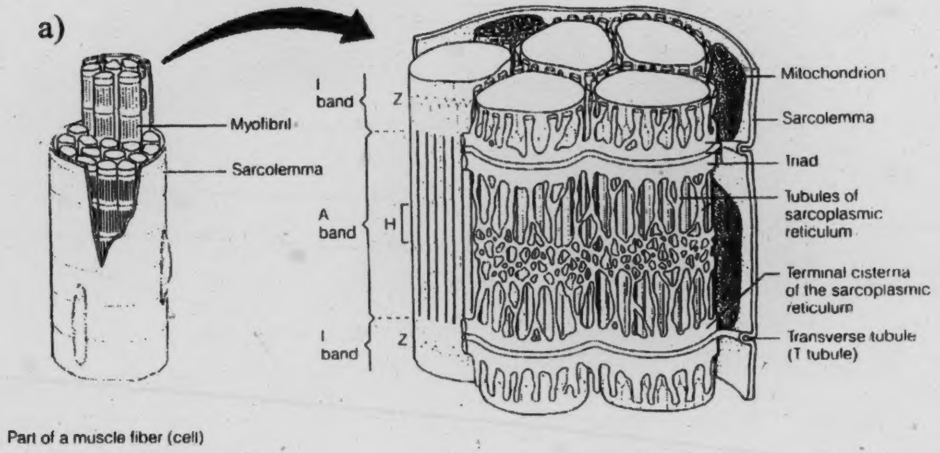
Figure 2.7 Summary diagram of biphasic myogenesis in the pig.

2.3.3. The cellular structure of skeletal muscle

The basic structural unit of the individual myofibre involved in contraction is called a sarcomere. Each sarcomere extends from one Z line to the next; the Z-disks mechanically link each sarcomere end to end. Thin filaments of the protein actin are attached to each Z line and these thin filaments also contain tropomyosin and troponin. Troponin is a regulatory protein, which contains Ca^{2+} binding sites that are involved in control of contraction and relaxation (Figure 2.8b).

Thick filaments are composed of myosin which is a large complex molecule consisting of tail, neck and head regions. The tails aggregate to form thick filaments, with the neck and head projecting laterally to form a crossbridge. Each head contains an actin-binding site and an enzymatic site called myosin or myofibrillar adenosine triphosphatase (mATPase) that can hydrolyse ATP to ADP and inorganic phosphate (Pi). The interactions between the crossbridges and the thin filament draw the thin filaments toward the centre of the sarcomere thereby causing shortening of the sarcomere as the Z-disks come closer together.

Different fibre types within the same or different muscles have different contractile properties, reflecting the fact that their sarcomeres contain different isoform combinations of myosin together with different isoform combinations of other structural and soluble muscle proteins. The different isoforms are generated either from separate genes or by differential splicing of the same gene (as reviewed by Buckingham, 1992).


a)
Myofibril
Sarcolemma
I band
Z
A band
H
I band
Z
Mitochondrion
Sarcolemma
Triad
Tubules of sarcoplasmic reticulum
Terminal cisterna of the sarcoplasmic reticulum
Transverse tubule (T tubule)
Part of a muscle fiber (cell)

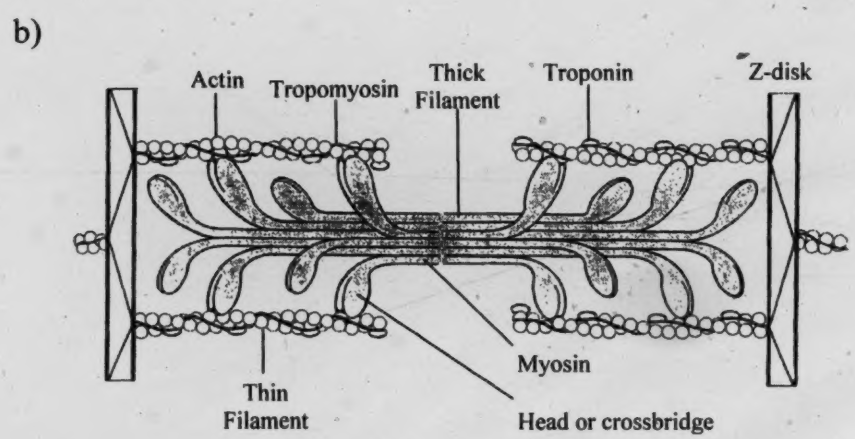

b)
Actin
Tropomyosin
Thick Filament
Troponin
Z-disk
Thin Filament
Myosin
Head or crossbridge

Figure 2.8 Skeletal muscle structure a) Relationship of the sarcoplacmic reticulum and T-tubules to the myofibrils of skeletal muscle (from Marieb, 1998) and b) Sarcomere structure (after Berne and Levy, 1990).

2.3.3.1 Myosin

Myosin is the primary component of muscle and is an asymmetric, hexameric protein consisting of four light chains (approximately 20 kDa each) and two heavy chains (approximately 200kDa each) (Pette and Staron, 1990 and references therein). Myosin exists in different isoforms and the type of myosin present in a muscle is of great physiological importance as it is correlated to the contractile properties of the fibre. Various studies have shown that heavy chains largely determine the level of enzyme activity of myosin and that light chains are probably not directly involved in the mechanism of action of the protein (Picard et al., 1994 and references therein). To date, the most informative methods to delineate muscle fibre types are based on specific myosin profiles, especially the myosin heavy chain (MHC) isoform complement.

2.3.3.1.1 Myosin heavy chains (MHC)

The two myosin heavy chains intertwine to form an α-helical coil or rod with a globular head region which is the site of mATPase activity and actin binding as discussed in section 2.3.3. A total of 11 MHC isoforms have been identified in adult extrafusal fibres (Pette and Staron, 2000). The major MHC isoforms MHCIβ, MHCIIa, MHCIId and MHCIIb are found in a variety of adult mammalian skeletal muscles, whilst the remaining fibre types appear to be expressed in a muscle-specific manner and include MHCII_{m}, MHC_{eom}, MHCI_{ton}, MHCIα, MHCIa, MHC_{emb}, MHC_{neo}, as summarised in Table 2.2.

Table 2.2 Myosin heavy chain isoforms identified in adult extrafusal fibres of mammalian skeletal muscles (from Pette and Staron, 2000).

Designation	Nomenclature	Muscle/fibre location
Fast-twitch	MHCIIb	Fibre types IIB, IIBD, IIAB
Fast-twitch	MHCIId	Fibre types IID, IIBD, IIDA
Fast-twitch	MHCIIa	Fibre types IIA, IIAB, IIDA, IIC, IC
Fast-twitch	MHC_{eom}	Extraocular and laryngeal muscles
Fast-twitch	MHCII_{m}	Masticatory muscles
Slow-twitch	MHCIβ	Fibre types I, IC, IIC
Slow-twitch	MHCIα	Extraocular, diaphragm, masseter muscles, fast-to-slow transforming fibres
Slow-twitch	MHCIa	Plantaris, soleus, slow-to-fast transforming fibres
Slow-twitch	MHCI_{ton}	Extraocular, laryngeal, and tensor tympani muscles
Embryonic	MHC_{emb}	Extraocular muscles
Neonatal	MHC_{neo}	Extraocular, masseter muscles

Collectively, the various methods used for fibre typing (discussed in section 2.3.4) have revealed the existence of muscle fibres containing either a single MHC isoform (pure fibre types) or those containing two or more isoforms (classified as hybrid fibre types). According to the four major MHC isoforms, the following pure fibre types exist: slow type I with MHCIβ, and three fast types, namely type IIA, with MHCIIa, type IID with MHCIId (MHCIId and fibre type IID are considered to be equivalent to MHCIIx and fibre type IIX, respectively; Pette and Staron, 1990) and type IIB with MHCIIb (Pette and Staron, 1990; Lefaucheur et al., 1995). The simultaneous expression of more than one MHC, results in the formation of hybrid fibres, which can be subdivided based on the predominant MHC isoform present. The co-expression of MHC types I and IIA resulting in the population of fibres histochemically classified as C fibres was elucidated by Staron and Pette (1986). Table 2.3 summarises the hybrid fibre types (as discussed by Pette and Staron, 2000). Coexpression of multiple MHC types within a fibre occurs under normal conditions and during fibre type transformation and increases the complexity of the fibre typing procedure.

Table 2.3 Summary of hybrid fibre types (as discussed by Pette and Staron, 2000).

Fibre type	Composition of MHC isoforms
Type I/IIA, also termed IC	MHCIβ > MHCIIa
Type IIA/I, also termed IIC	MHCIIa > MHCIβ
Type IIAD	MHCIIa > MHCIId
Type IIDA	MHCIId > MHCIIa
Type IIDB	MHCIId > MHCIIb
Type IIBD	MHCIIb > MHCIId

The MHC complement of a fibre is strongly correlated to its contractile properties. Pette and Staron (2000) highlight the existence of a strong correlation between MHC complement and stretch-activation kinetics. Velocity of contraction was lowest in type I fibres and increased in the order IIA < IID < IIB. Values obtained from hybrid fibres fell between the pure fibres and were spread out according to the specific ratios of the coexisting MHC isoforms.

Species differences exist in the expression of some of the MHC isoforms. Body size appears to correlate with the relative concentrations of MHCIβ, MHCIIa, MHCIId/x and MHCIIb, as body mass increases, expression levels of the slower isoforms increase at the expense of faster isoforms (Hämäläinen and Pette, 1995). Muscles in the human do not appear to express MHCIIb under normal conditions, therefore fibres that had previously been classified as type IIB in human muscle have been renamed type IID based on their MHC complement that resembles MHCIId/x of the rat (Pette and Staron, 2000). In addition to their expression in specific adult mammalian muscles such as the extraocular and masseter muscles, the developmental isoforms are expressed in embryonic and neonatal mammalian skeletal muscles. The remaining fast and slow heavy chains are expressed in a variety of muscles of different species. Fast twitch MHC_{eom} has been observed only in the extraocular muscles of mammals while MHC specific to tensor tympani jaw muscles of carnivore species have been identified (see references cited by Pette and Staron, 1990). The percentage of hybrid fibres, i.e. fibres co-expressing two or more myosin heavy chains, appears to vary in different species and increases under conditions of induced fibre transformation.

2.3.3.1.1.1 Myosin isoform transitions

Staron et al. (1983) and Pette and Staron (1990, 2000) review evidence for the continuum that exists between muscle fibre types. Fibres are dynamic structures, capable of altering their phenotype under various conditions. For example, changes in neuromuscular activity, mechanical loading or unloading, and altered hormone profiles and aging. The change in myosin isoforms tend to follow a general scheme of sequential and reversible transitions from fast-to-slow and slow-to-fast: (slow) MHCIβ ↔ MHCIIa ↔ MHCIId ↔ MHCIIb (fast).

Transitions in both myosin isoforms and fibre types can be achieved experimentally by the use of chronic stimulation of muscle fibres which mimics the tonic low-frequency impulse pattern normally delivered to a slow-twitch muscle (Salmons and Vrbová, 1969). In the rabbit, chronic low frequency stimulation (CLFS) induced a more pronounced fast-to-slow fibre transition compared with species such as the rat or mouse (Pette and Staron, 2000). The extent of fibre transition is dependent on the pattern and duration of stimulation. Transformation is reversible, since the fast fibre population is restored following cessation of chronic stimulation. Similar to CLFS, stretch and mechanical loading cause fast-to-slow transitions, while mechanical unloading (immobilisation of the muscle in a shortened position, hindlimb suspension or microgravity) induces slow-to-fast transitions, the extent of change depending on the initial proportion of fibre type in each muscle. Recently, Pette et al. (2002) investigated the effect of CLFS of rat muscles. Although CLFS of normal rat fast-twitch muscles induces sequential transitions in MHC expression from fast-to-slow, the final step of the transition (the upregulation of MHCI) was previously observed only after extremely long stimulation periods. However, results suggested that satellite cells and/or regenerating fast rat muscle fibres are capable of switching directly to a slow program under the influence of CLFS and therefore, appear to be more malleable than adult fibres. Pette and Staron (2000) also review the effects of hormones and aging on fibre type transitions. Thyroid hormone appears to have the greatest effect on muscle fibre phenotype (hypothyroidism causes fast-to-slow transitions, while hyperthyroidism elicits transitions in the reverse direction) as discussed in section 2.3.5.3. In addition to muscle atrophy, it has been suggested that aging causes fast-to-slow transitions (as reviewed by Larson and Ansved, 1995).

2.3.3.1.1.2 Mechanisms involved in fibre type transitions

Pette and Staron (2000) briefly discussed possible mechanisms involved in transition of fibre types. Fibre type transformation clearly represents a highly coordinated process of upregulation and downregulation of genes since the isoform profiles of a multitude of sarcomeric proteins are altered, in addition to changes in myosin expression. Transcription, translation and proteolysis are involved in the exchange of protein isoforms. CLFS has been shown to increase intracellular Ca^{2+}, which is of interest with regard to its role as a second messenger in the control of gene expression. In addition, the calcineurin-dependent pathway has been implicated in the control of gene expression in fast and slow muscle fibres. Olson and Williams (2000) and Carpenter (2001) reviewed the role of calcineurin in the remodelling of skeletal muscles. Calcineurin is a Ca^{2+}, calmodulin-dependent serine-threonine specific protein phosphatase, localized to the cytoplasm. Calcineurin is ubiquitous but is present at about 10-fold higher concentrations in brain and muscle than in other cell types. Calcineurin exists as a heterodimer, composed of a catalytic (CnA) and regulatory subunit (CnB).

Briefly, calcineurin is activated by binding of intracellular Ca^{2+} to regulatory subunit CnB, which induces a conformational change in calcineurin, which exposes calmodulin binding sites on CnA. At the same time, Ca^{2+}-bound calmodulin binds to calcineurin regulatory subunit CnB, exposing Nuclear factor of activated T-cells (NFAT)-binding sites. Activated calcineurin dephosphorylates NFAT. The Calcineurin-NFAT complex is translocated into the nucleus where the complex associates with other transcription factors such as myocyte-specific enhancer-binding factor 2 (MEF2) to promote transcription of fibre-type-specific and hypertrophy-inducing genes in skeletal myocytes.

More recently, Pallafacchina et al. (2002) presented the first evidence that fibre type specification and skeletal muscle hypertrophy are regulated by nerve activity through different molecular mechanisms. Whereas fibre type transitions were mediated through calcineurin and Ras-mitogen-activated protein kinase (Ras-MAPK) pathways, muscle fibre size was shown to be regulated by the activity of phosphoinositide 3-kinase (PI3K), its downstream target, the serine-threonine protein kinase-B (PKB) and downstream effector mammalian target of rapamycin (mTOR). Activation of the PI3K-PKB-mTOR pathway was reported to increase fibre size and prevent denervation atrophy in regenerating and adult rate muscles but did not affect fibre type.

2.3.3.1.2 Myosin light chains (MLC)

The four myosin light chains are associated with the two myosin heads. The role of the light chains is poorly understood but they may be involved in modulating interactions between myosin and actin (Pette and Staron, 1990 and references therein). The light chains are separated into two classes: phosphorylatable light chains and the alkali light chains. The different combinations of MLC and MHC result in the various isoforms of myosin (each isomyosin is composed of two MHC, two phosphorylatable MLC and two alkali MLC (Talmadge et al., 1993).

2.3.4 Muscle fibre classification

The diversity of actions performed by skeletal muscles throughout the body requires that the muscles used to perform them have different properties. Some muscles are called upon to maintain a high level of tension for long periods of time without fatigue, for example, the postural soleus muscle that contains predominantly slow-contracting muscle fibres. The extraocular eye muscles are required to produce intermittent rapid movements and contain predominantly fast-contracting muscle fibres. Muscles that have to perform both endurance and rapid actions have a more even mixture of these fibre types.

The overlap of various characteristics, including MHC content between muscle fibre types, contributes to the incompatibility of the different classification schemes used for muscle fibre typing (section 2.3.4.2). Pette and Staron (1990) stress the importance of referring to the method used for fibre typing when making a distinction between specific fibre types.

2.3.4.1 Methods of muscle fibre typing

The number of fibre types that can be differentiated depends on the method of fibre typing used. Various methods exist for the purpose of classifying muscle fibre types, from the traditional histochemical methods to more recent techniques such as immunohistochemistry, enzyme-linked immunosorbant assay (ELISA) and gel electrophoresis, which have offered new perspectives for muscle fibre typing. The various methods used for fibre typing have been reviewed by Peter et al. (1972), Pette and Staron (1990), and Talmadge et al. (1993). The study of muscle fibre types has been a subject of interest for well over a century. The diversity of skeletal muscle fibres was recognized as early as 1873 when Ranvier distinguished "white" and "red" muscles. Muscles that were slow contracting appeared red whereas fast contracting muscles appeared white. Further studies towards the end of the 19th Century led to observations of muscle fibre size and translucency. However, it was not until the mid 20th century that a breakthrough in the description of fibre types resulted from the combination of new histological and physiological methods and from the histochemical studies of the 1940s and 1950s (Padykula and Herman, 1955).

As one of the earliest and simplest classifications, skeletal muscle fibres were classified into two main types using histochemical methods, Type I (slow) and Type II (fast), depending on their differing contractile properties. However, this has subsequently been shown to be a gross oversimplification, as fibre types also differ by their relative proportions of mitochondria and respiratory enzymes, which determine the metabolic properties of the muscle.

One of the most well known fibre classification systems is that of Brooke and Kaiser (1970a, 1970b), who developed a histochemical method based on mATPase activity. Although only a small number of major fibre types can be determined using this qualitative histochemical method, the procedure is still extensively used in physiological studies to identify the composition of muscle fibres. Brooke and Kaiser (1970a, 1970b) proposed a three pH range classification system of muscle fibres based on their pH sensitivity with the histochemical myosin ATPase reaction. As explained in section 2.3.3, the mATPase enzyme complex is located at the head of the myosin heavy chain, its function to catalyze the breakdown of ATP to ADP and inorganic phosphate (P_i). The activity of mATPase is directly related to the speed of muscle fibre shortening and has been shown to vary between fast and slow twitch muscle fibres. The Brooke and Kaiser technique is based on the fact that the mATPase systems, which show differing activities in different muscle fibre types, also have different pH sensitivities. The mATPase systems have different catalytic properties due to differences in amino acid structure and binding properties of the active site of the enzyme. The different amino acid sequences may bind to free hydrogen ions (H^+) with differing affinities and therefore cause the different mATPase systems to respond differently to varying pH levels. The mATPase system associated with type I fibres is more alkali-labile than the mATPase system associated with type II fibres which are alkaline stable and acid-labile. Fibre types I, II, IIA, IIB and IIC can be distinguished based on fibre staining intensity at differing pH conditions (see Table 2.2). Meijer (1970) and Guth and Samaha (1969, 1970) and Samaha et al. (1970) also presented a method for the histochemical demonstration of mATPase activity, which differ in minor respects to the Brooke and Kaiser method.

Table 2.4 Muscle fibre nomenclature according to Brooke and Kaiser (1970a, 1970b)

Fibre type	Contraction speed	Nomenclature
I	Slow twitch	Slow oxidative
IIA	Fast twitch	Fast Oxidative Glycolytic
IIB	Fast twitch	Fast Glycolytic
IIC	Unclassified	

Type I and II fibres can be classified independently of mATPase histochemistry by their specific enzyme activity profiles. In this context, activity ratios between enzymes of anaerobic and aerobic pathways can be used as discriminative parameters (Pette and Spamer, 1986). Peter et al. (1972) analyzed certain guinea pig and rabbit skeletal muscles that were composed solely or predominantly of a single fibre type for various enzymatic and substrate characteristics. Results showed that the fibre types could be conveniently classified into three categories based on fibre contraction time, and glycolytic and oxidative capacity. They examined anaerobic (glycolytic) capacity as indicated by various factors including glycogen concentration and phosphorylase, lactate dehydrogenase and mitochondrial α-glycerophosphate dehydrogenase activities. Aerobic (oxidative) capacity was examined by studying cytochrome concentration and succinate dehydrogenase activity. The study led to the division of slow twitch oxidative, fast twitch oxidative glycolytic (FOG) and fast twitch glycolytic (FG) fibres, which were suggested to correspond with Brooke and Kaisers', Type I, Type IIA and Type IIB fibres respectively. Peter et al. (1972) correlated the histochemical characteristics of fibres found in their study with an outline of previous terminologies employed to describe various types of fibres.

In many instances, immunohistochemical determination of muscle fibre types using monoclonal antibodies against different isoforms of myosin heavy and light chains may be a more suitable technique for the qualitative determination of muscle fibre type characteristics. Although conventional histochemical techniques are well suited to resolve most adult fibre types (I, IIA and IIB) in mature muscle, they may not allow clear classification during the fetal and perinatal periods. During this time, the actomyosin ATPase is generally more or less resistant to both acid and alkaline pH and many fibres behave in the same manner as fibres containing a mixture of adult fast and slow myosins, and as a consequence, have often been classified as type IIC fibres (Lefaucher et al., 1995). However, these fibres have been shown to contain different MHC isoforms such as embryonic and fetal isoforms (Pette and Staron, 1990). Immunohistochemical techniques allow classification of fibre types during the fetal and perinatal period using antibodies raised against fetal myosin and specific for fetal MHC (Lefaucher et al., 1995). Immunohistochemical techniques depend on the availability of specific antibodies raised against specific antigens. Most available specific antibodies raised against different MHCs have been successfully used in rat tissue (Schiaffino et al., 1989); however, specificity of many antibodies in other species remain to be documented. Draeger et al. (1987) used monoclonal antibodies to MHCs to describe and define the appearance and maturation of myotubes in developing human quadriceps muscle. Fazarinc et al. (1995) and Lefaucheur et al. (1995, 1997, 2001, 2002) used monoclonal antibodies for porcine myosin heavy chains and the Labelled Streptavidin Biotin indirect method for immunohistochemical staining. Determination of muscle fibre number by histochemical and immunohistochemical methods is very tedious and laborious, even if modern digital imaging techniques have made significant contribution to reducing the labour involved. As a consequence, recent studies have shown increased focus on new technologies to study muscle fibre types. Myosin isoforms can also be separated based on their electrophoretic mobility using standard or gradient SDS-PAGE (Bee et al., 1999; Lefaucheur et al., 2001), by ELISA (Barrey et al., 1995) or by a combination of *in situ* hybridisation and immunohistochemistry (Lefaucheur et al., 1997, 1998, 2002; Da Costa et al., 2002).

Although a wide variety of muscles have been used for studies of fibre typing, some are more popular than others. A large number of studies carried out in the pig have focussed on the *semitendinosus* (ST) muscle, which is one of the hamstring muscles of the hindlimb. The ST has been used for a number of reasons. Firstly it was shown to be a good indicator of whole body muscling (Mulvaney et al., 1985). Additionally, no evidence was found that the ST contains intrafasicularly terminating fibres (Wigmore and Stickland, 1983) which could bias counts of muscle fibre number (Swatland and Cassens, 1972). Finally, the numbers of muscle fibres remains constant for the ST muscle with increases in age and body weight, supporting the general consensus that muscle fibre number is fixed at birth in the pig (Handel and Stickland, 1987).

2.3.4.2 Compatibility of classification schemes

The compatibility of different classification schemes is an important factor, which further complicates the muscle fibre typing issue. Staron et al. (1983) examined the ultrastructural characteristics of muscle fibres characterized as Types I, IIA, IIAB and IIB using the mATPase technique. They found the volume percent mitochondria to be significantly different between all fibre types, and found additional differences in lipid volume. Ultrastructural data revealed considerable overlap between the fibre types for metabolic parameters, leading the authors to suggest the existence of a continuum of muscle fibre types based on ultrastructural characteristics in addition to metabolic enzyme classification. Furthermore, muscle fibres may transform in response to the amount and type of usage. Various studies have been carried out comparing different classification systems (Nemeth et al., 1979; Bee et al., 1999). Some have been found to give consistent results, whilst others are clearly not equivalent.

As discussed in section 2.3.3.1.1.1 muscle fibres are versatile structures, which adapt to altered functional demands, hormonal signals and changes in neural input. The dynamic nature of muscle fibres makes it difficult to separate them into distinct categories. Some molecular or functional properties of muscles may change without affecting others or changing the histochemical appearance of a given fibre (due to muscle or species specificity, developmental or adaptive processes or pathological conditions), resulting in the existence of fibre type transients. For these reasons, a distinction between specific types of fibres should refer to the method upon which it is based. Applying any type of rigid structural and/or functional classification scheme is an oversimplification; however, fibre type classification systems have helped to define functional, metabolic and molecular properties of muscle fibres.

2.3.5 Regulation of myogenesis

The main events in myogenesis are regulated during prenatal and postnatal life by intrinsic and extrinsic factors. Intrinsic factors include the myogenic regulatory factors (MRFs) in particular, MyoD, Myf5, myogenin and MRF4 (also known as Myf 6), thyroid hormones and growth factors. Extrinsic factors include environmental influences such as nutrition, breed and genotype. A plethora of information on the regulation of myogenesis has accumulated since the early 1990s. However, some of the better and more up to date reviews include Maltin et al. (2001), Wigmore and Evans, (2002), Picard et al. (2002) and Parker et al. (2003). In the absence of a definitive review that encompasses all molecular signals and factors suggested to be involved in myogenesis, a brief summary of these reviews is presented in the following paragraphs.

Discussion of the processes leading to the formation of myogenic cells uses a variety of different terms including commitment, specification, determination and differentiation. Since slight differences exist in the terminology used in the published literature, for the purposes of this chapter, the definitions used by Parker et al. (2003) will be adopted. Commitment refers to the restriction of cells to a particular developmental fate before differentiation. Specification is the process by which cells acquire a fate (what a cell and its progeny will give rise to in a later stage of development). Determination is the process of irreversible specification, whereby a cell becomes able to differentiate autonomously, even if placed in another part of the embryo. Differentiation is the formation of a specialized cell type by a process that is not normally reversible.

2.3.5.1 The embryonic origin of skeletal muscle

One of the earliest steps in the development of vertebrate embryos is somitogenesis. Embryonic skeletal myogenic cells arise from somitic progenitors in response to signalling molecules from adjacent structures. Figure 2.9 illustrates the structures involved in early muscle development in the embryo.

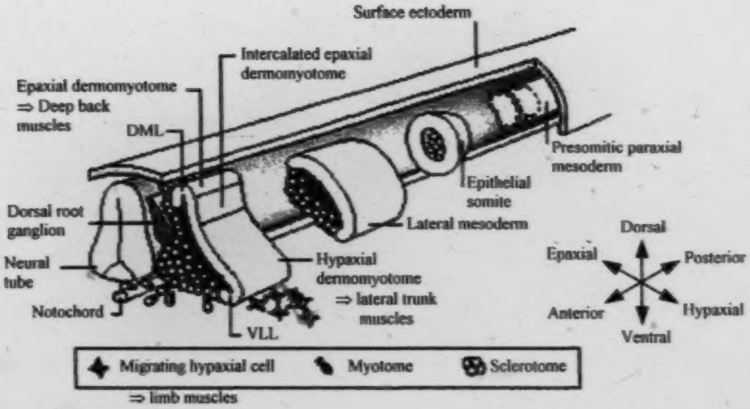

Surface ectoderm
Intercalated epaxial dermomyotome
Epaxial dermomyotome ⇒ Deep back muscles
DML
Presomitic paraxial mesoderm
Epithelial somite
Dorsal root ganglion
Lateral mesoderm
Dorsal
Epaxial
Posterior
Neural tube
Hypaxial dermomyotome ⇒ lateral trunk muscles
Anterior
Hypaxial
Notochord
VLL
Ventral
Migrating hypaxial cell
Myotome
Sclerotome
⇒ limb muscles

Figure 2.9 The embryonic origin of limb and trunk skeletal muscle slightly modified from Parker et al. (2003).

The notochord is a cylindrical cord of cells on the dorsal aspect of an embryo marking its longitudinal axis. The notochord is surrounded on either side by the presomitic paraxial mesoderm which gives rise to the somites. Somite formation occurs along the dorsal-ventral axis in the rostral to caudal direction (back to front surface in a head to tail direction) by segmentation of the paraxial mesoderm in response to signals from the notochord and the neural tube (the neural tube is the precursor of the CNS in the embryo and is formed by the invagination and fusion of the neural plate which is the thickened ectoderm dorsal to the notochord). The ventral region of differentiating somites gives rise to the sclerotome, from which the ribs and vertebrae form, whilst the dorsal region of the somites forms the dermomyotome. The dermomyotome is further subdivided into the hypaxial region from which the lateral trunk muscles form and the epaxial dermomyotome, which gives rise to the deep back musculature. The cells of the dorso-medial lip (DML) and ventro-lateral lip (VLL) migrate under the dermomyotome and terminally differentiate to form the epaxial and hypaxial myotome respectively. Subsequently, cells of the VLL develop and migrate to sites of later limb formation.

2.3.5.2 Muscle specific transcription factors

As reviewed by Parker et al. (2003), the MRFs play a role in skeletal muscle development and differentiation and include Myf5, MyoD, myogenin and MRF4 (also known as Myf 6). The four MRFs each contain a basic helix-loop-helix (bHLH) domain, which is required for DNA binding and dimerization with the E-protein family of transcription factors. In order to bind to their target sequences with high affinity and activate transcription, MRFs form heterodimers with members of the bHLH protein family such as E12 and E47. As reviewed by Parker et al. (2003), the heterodimers then bind to a DNA control element known as the E-box consensus within muscle-specific gene promoters. In addition to the MRFs, the myocyte-specific enhancer-binding factor-2 (MEF2) family of transcription factors is involved in the activation of muscle-specific gene expression (see Parker et al., 2003 and references therein).

Genetic studies of MRFs using gene knockout mouse models have been the major route through which the functions of the MRFs have been elucidated. The scope of this chapter does not allow an in depth review of these studies, however the reviews of Pownall et al. (2002) and Parker et al. (2003) provide a detailed summary. Briefly, the essential role for MyoD and Myf5 in myogenic specification was illustrated using mice that lacked both MyoD and Myf5. These embryos had a complete lack of skeletal muscle and died at birth from respiratory failure. Once myogenic cells have undergone specification, the development of skeletal muscle requires the differentiation and fusion of muscle progenitor cells to form multinucleated myotubes. Mice carrying null mutations for myogenin died at birth as a result of the absence of skeletal muscle, although they had a normal number of undifferentiated myoblasts. Therefore, myogenin is understood to play a role in differentiation rather than specification. MRF4 null mutant mice show reductions in skeletal muscle, although deficiencies were less severe than observed in myogenin double mutants, MRF4 clearly plays a role in terminal differentiation of muscle.

The MRFs are activated and regulated by a complex cascade of signalling molecules, the detailed description of which is beyond the scope of this chapter. Briefly, there are a number of signals from the neural tube and notochord that induce expression of Myogenic regulatory factors or MRFs (as discussed by Maltin et al., 2001; Pownall et al., 2002; Parker et al., 2003). The pathways that regulate Myf5 and MyoD expression are presented in detail by Parker et al. (2003). Myf5 expression is positively regulated by Sonic Hedgehog (Shh), the Wingless (Wnt1) pathway, via the Frizzled (Fzd) receptor, and MyoD. The expression of MyoD is in turn activated by Myf5, Wnt7a, via a β-catenin independent pathway and Wnt3 acting through a β-catenin/T-cell factor (Tcf) pathway. The paired-domain transcription factor Pax3 also has a role in the regulation of Myf5 and MyoD expression. Protein kinase C (PKC), fibroblast growth factor (FGF), transforming growth factor β (TGFβ), oncogene products such as c- *fos* and c-*jun* and tissue specific transcription factors such as MEF2 can also affect the activity of the MyoD family by promoting cell division (Dauncey and Gilmour, 1996).

Somitic myogenesis is also under negative regulation via a number of different factors. Expression of Myf5 and MyoD is inhibited by the bone morphogenic proteins (BMPs), which are members of the TGF family of signalling molecules. BMP signalling may in turn be modulated through the action of the BMP antagonist Noggin. Myostatin, a member of the TGFβ superfamily, has also been shown to be a negative regulator of myogenesis and functions by inhibiting myoblast proliferation (Thomas et al., 2000; Ríos et al., 2002). Belgian Blue and Piedmontese breeds of cattle, which are characterised by an increase in muscle mass (double-muscling), have mutations in the myotstatin coding sequence and have been the focus of a number of studies (as discussed by Lee and McPherron, 1999; Kocamis and Killefer, 2002). Shaoquan et al. (1998) examined the expression of myostatin in porcine tissues and noted that myostatin expression in skeletal muscle peaked prenatally (detected in whole fetuses at 21, 35 and 49 days and was highest at 105 days of gestation, declining after birth). Greater expression of myostatin was shown to be associated with low birth weight.

The soluble Frizzled related proteins (sFRP) bind to Wnts, thereby preventing the expression of Myf5 and MyoD. The Notch signalling pathway also negatively regulates muscle differentiation by inhibiting the expression of MyoD. In addition to the signalling molecules discussed above, Dauncey and Gilmour (1996) discussed the role of negative regulators of gene expression, in particular, the inhibitor of differentiation (Id) family of bHLH proteins. Id proteins lack a DNA binding domain, but can form heterodimers with members of the E-proteins family (Langlands et al., 1997) thereby preventing them from binding to a MyoD protein. Levels of Id protein are repressed during myogenesis, but have been shown to increase during muscle atrophy (Dauncey and Gilmour, 1996 and references therein). The MyoD family and Id proteins work in concert to regulate muscle fibre size both pre and postnatally. Melnikova et al. (1999) examined and compared the biological activities of four members of the Id family. They noted differences in affinity for MRFs and suggested that members of the Id family might be involved in the regulation of distinct developmental pathways.

The restriction of MRF expression, and resulting inhibition of differentiation via these negative regulators is vital to allow a sufficient number of muscle precursor cells to migrate to the myotome and limb buds before they undergo differentiation. Clearly, the temporal expression of the various signalling molecules and MRFs must be tightly controlled to ensure the appropriate expansion and differentiation of myogenic cells

Collectively, results indicate that Myf5 and MyoD are essential regulators for specification of muscle progenitors. Myf5 controls early processes of progenitor specification, including cell proliferation and local cell migration to establish progenitor stem cell pools at sites of myogenesis, whereas MyoD functions in the initiation of differentiation and contractile protein gene activation. Myogenin and MRF4, are differentially expressed in differentiated muscles, consistent with their later regulatory functions in processes of muscle differentiation, regeneration and fibre type specification. Myogenin and MRF4 likely regulate contractile protein target genes including those involved in fast and slow fibre differentiation and regeneration. The specific muscle gene targets of Myogenin and MRF4 remain to be identified (Pownall et al., 2002).

Postnatally, less is understood regarding the possible roles of these transcription factors and signalling cascades in the regulation of muscle development. Satellite cells are mononucleated, quiescent muscle precursor cells, located between the basal lamina and the sarcolemma of adult skeletal muscle fibres, which are thought to be involved in growth and regeneration of muscle (as reviewed by Campion, 1984; Bishoff, 1994;

Schulz and McCormick, 1994; Bornemann et al., 1999). Dormant satellite cells do not express MRF transcripts or proteins (as discussed by Putman et al., 2000; Parker et al., 2003). However, once satellite cells are activated, they are committed to the myogenic lineage as myogenic precursor cells (MPC) and upregulate Myf5 and MyoD. Parker et al. (2003) suggested that adult stem cells might differentiate directly as MPCs in response to molecules such as the Wnts or Shh, which have been shown to have a role in embryonic myogenesis. MPC proliferation might be maintained through the action of BMP or the Notch cascade that inhibits the expression of MyoD.

2.3.5.3 Thyroid hormones

Thyroid hormones (TH) are the most studied hormones with respect to muscle development. They stimulate conversion of slow to fast myosin heavy chain isoforms, increase the abundance of mitochondria and their respiratory enzymes, and increase the concentration of Na^+, K^+-ATPase and Ca^+-ATPase (as reviewed by Dauncey and Gilmour, 1996). It has been hypothesized that TH directly regulate both the MyoD family of transcription factors and contractile protein genes themselves. The actions of TH are mediated by interaction with specific DNA sequences, termed TH response elements which are usually located in the 5' promoter region of target genes (Dauncey and Gilmour, 1996 and references therein).

The relative importance of TH in early embryonic and fetal development of muscle has yet to be clearly established. Butler-Browne et al. (1990) concluded that in humans, excessive amounts of thyroid hormone resulted in precocious accumulation of the adult MHCs, accompanied by a precocious maturation of the muscle.

Perinatal development in mammals is associated with marked changes in circulating concentrations of TH, and these changes in TH status may have a role in regulating muscle development in the perinatal period. D'Albis et al. (1987) studied the regulation by TH of terminal differentiation of murine skeletal muscle. Hypothyroidism of newborn mouse pups completely inhibited postnatal muscle differentiation. In contrast, hyperthyroidism significantly accelerated myosin transition and the switch in the myofibrillar pattern. The authors suggested a role for TH in regulating the appearance of myosin and adult fibre types and in modulating the disappearance of neonatal fibre types.

Berthon et al. (1993) carried out a study to determine the effects of hypothyroidism during late fetal life in pigs on the pattern of plasma TH levels (T_3 and T_4) and on the early postnatal development of thermoregulation. The results of their study suggested a major role for thyroid hormones in the control of thermogenesis in the newborn pig. Postnatally, it is well established that TH play a central role in controlling the switching between mature myosin heavy-chain isoforms, with hyperthyroidism inducing a transition from slow to fast isoforms, and hypothyroidism inducing an increase in proportion of slow fibres (Pette and Staron, 2000).

2.3.5.4. Other hormones and growth factors

Other hormones such as glucocorticoids, insulin and GH may also be involved in regulation of muscle development, although their precise roles remain to be established. It has been shown that myosin heavy chains can be affected postnatally by each of these hormones (Dauncey and Gilmour, 1996). IGFs and their type 1 receptor play a critical role not only in the early stages of myogenesis at the level of stem cell proliferation, but also in terminal differentiation of myoblasts into myotubes (Florini et al., 1991; Kou and Rotwein, 1993). The levels of IGF-I and IGF-II mRNA in fetal tissues are increased in muscle at the time when secondary fibre formation is taking place, these high levels of IGF may play a key role in prenatal muscle development (as reviewed by Dauncey and Gilmour, 1996). Postnatally, IGFs almost certainly play a role in fibre hypertrophy (Devol et al., 1990). FGF and TGF-β are potent inhibitors of differentiation. As discussed in section 2.3.5.2, they also appear to act by altering gene expression of the MyoD family of regulatory proteins (Dauncey and Gilmour, 1996).

2.3.6 Prenatal muscle development in the pig

In addition to the intrinsic factors that affect myogenesis discussed in section 2.3.5, extrinsic factors that influence prenatal events in muscle development include nutrition, breed and genotype. Prenatal events may also contribute to the rate of postnatal growth (section 2.3.7) particularly with respect to the occurance of IUGR. Furthermore, a number of studies have shown that muscle growth processes are related to meat quality traits (section 2.3.8).

A number of early studies examined the effects of IUGR on fetal muscle development. The brain sparing effect seen to occur in cases of IUGR, produced by the preferential growth of the brain at the expense of other organs including the muscle was explained in section 2.3.1. Widdowson (1971) studied the effects of growth retardation on various aspects of body composition in the pig and demonstrated that the muscles of 'runt' or growth restricted pigs were smaller and contained less DNA than fetal organs including the brain and heart. The quadriceps muscles grew approximately in parallel with the body as a whole and were therefore smaller in growth-restricted animals. In an experiment that only compared two animals, the postnatal growth rate of a low birthweight pig was also shown to be slower than that of its larger sibling. The low birthweight pig failed to attain the same adult size even when fed *ad libitum*. The effect of runting on postnatal development has however been examined with greater numbers of animals in subsequent studies where similar results were observed. The effects of prenatal runting on subsequent postnatal development were studied by Hegarty and Allen (1978), and Powell and Aberle (1980, 1981). Both studies concluded that runt pigs had smaller muscles than their larger littermates and grew more slowly and less efficiently. Taken together, these results suggested that prenatal factors, which may affect total fibre number in a given muscle, could have permanent effects on the postnatal growth of muscles in pigs.

The relative effect of maternal undernutrition or growth retardation *in utero* on the number of primary fibres and secondary myotubes is a critical issue, and will determine postnatal growth potential. In particular, the reduction in the number of secondary fibres is responsible for most of the variation in muscle development seen within litters. Wigmore and Stickland, (1983) studied muscle development in large and small pig fetuses. The time of formation of primary and secondary fibres and the number of primary fibres formed were the same in both large and small littermates. However, the number of secondary fibres formed was lower in the smaller fetuses and this resulted in a 17% difference in total fibre number at birth. Primary fibres in the small fetuses were smaller and it was suggested that this might have restricted the available surface area for secondary fibre formation.

Stickland and Handel (1986) then investigated whether genetically small animals developed fewer muscle fibres in their muscles by the same mechanism as resulting from nutritional deprivation. This was shown not to be the case. The numbers, ratios and distributions of muscle fibre types were investigated in Large White and miniature pigs. Total myofibre number and total primary myofibre numbers in the *semitendinosus* muscle were greater in Large White than in miniature pigs (173 and 115% respectively). Rather than a reduction in the number of secondary fibres forming around each primary, as in the case of nutritionally-small pigs, the number of primary fibres which each formed their own metabolic bundle (single primary fibre surrounded by secondary fibres) was considered to be the most important factor responsible for the overall difference in myofibre number. It was concluded that different mechanisms result in decreased muscle fibre development of genetically small animals compared to animals subjected to nutritional deprivation *in utero*.

Handel and Stickland (1987) determined that low birth weight in the pig was associated with a permanently reduced total numbers of fibres in runt littermates. A reduced muscle fibre number was not always associated with low birth weight; however, when this was the case, it was generated through a reduced secondary to primary fibre ratio. Primary fibre number was not significantly affected in low birth weight pigs except in extreme cases of runting. Dwyer and Stickland (1991) demonstrated that primary fibre number also varied between litters, and was responsible for the variation in total muscle fibre number between litters. Taken as a whole, the authors concluded that the results of these two studies suggested that primary fibre number is a relatively fixed genetic component compared to secondary fibre number, and is therefore more indicative of the genotype of an animal with respect to muscle. Secondary fibre number, although having a genetic component, is more vulnerable to environmental factors *in utero*.

Ward and Stickland (1991) and Dwyer and Stickland (1992) also studied the effect of maternal undernutrition in the guinea pig to determine if the anatomical location of a muscle affected the influence of undernutrition on muscle fibre number. Nutritional restriction had a more detrimental effect on the *biceps brachii* muscle (predominantly fast twitch fibres) than the *soleus* muscle (predominantly slow twitch fibres), leading to the conclusion that fast muscles suffered a disproportionate reduction in fibre number due to their relatively high secondary fibre population. Slow muscles, with a greater proportion of primary fibres, were less affected by undernutrition. The effect of undernutrition on muscle fibre number seemed to be a function of the fibre types in that muscle, rather than the anatomical location of the muscle. A subsequent study in the guinea pig by Dwyer et al. (1995) further elucidated the timing and consequences of maternal feed restriction during gestation on fetal muscle fibre development. This study provided further evidence for the hypothesis that undernutrition exerts its main effects on placental development, and that secondary fibre proliferation is indirectly affected owing to impaired placental nutrient transfer (as discussed in section 2.2.2.3).

Faster growing strains of many animals, including pigs, tend to have more muscle fibres than do slower-growing strains, as discussed by Dwyer et al. (1993). These authors suggested that pig birth weight was a good indicator of the early growth rate of pigs, and that muscle fibre number was a more important determinant of postnatal growth after 10 weeks of age, such that littermates with a higher fibre number grew faster and more efficiently than littermates with a lower fibre number.

The critical time period for formation of secondary muscle fibres was highlighted in a study by Dwyer et al. (1994), who tested the hypothesis that increasing third parity sow feed intake at specific times during gestation would result in an increase in muscle fibre number in the fetuses, by affecting the developing population of secondary fibres. Since it was previously demonstrated that secondary fibre hyperplasia begins at approximately day 50 of gestation in the pig and continues until approximately day 90 (Wigmore and Stickland, 1983), three time periods were examined in this study. The first time period was up to the onset of secondary fibre hyperplasia (gestational day 25 to 50), the second time period covered the time of fibre hyperplasia (day 50 to 80) and the third period covered both developmental processes (day 25 to 80). The study demonstrated that increased maternal feed intake occurring immediately before muscle fibre hyperplasia (day 25 to 50) increased the production of secondary myofibres in lighter weight pig fetuses. Primary fibre number was unaltered by nutritional manipulations, possibly due to the fact that primary fibres form relatively early in gestation, when the litter is making a negligible nutritional demand on the sow. Furthermore, the increased muscle fibre number had a significant effect on growth rate and feed conversion efficiency in the later stages of pig growth to 80kg, leading the authors to conclude that nutritional manipulations during early pregnancy have a permanent effect on the development of the placenta, thereby affecting fetal IGF levels with consequent effects on the developing muscle.

In contrast to Dwyer et al. (1994), Nissen et al. (2003) showed that *ad libitum* feeding compared with restrictive feeding of pregnant sows in early to mid-gestation had no beneficial effects on muscle fibre characteristics or DNA and RNA content of the offspring at slaughter. Neither were there any beneficial effects on postnatal growth characteristics and meat quality traits. The authors suggested that the level of feed restriction might affect the traits observed, in addition to the time period of treatment during gestation. Species differences were also likely, especially in relation to the number of fetuses per parity, with littermates probably being more prone to undernutrition *in utero* due to competition for nutrients than would be a singleton fetus. Also, variation in litter size within a species may influence the variation in muscle fibre number and the response to nutrient restriction. Indeed, the authors observed a negative correlation between the number of pigs born per litter and the number of muscle fibres. The authors also speculated that the increase in sow body weight, with no effect on offspring birth weights, could be due to the possibility that high feed intake caused hormonal changes in the dam, so that nutrients were directed toward maternal tissues instead of fetal tissues. This suggestion is consistent with the results of studies of fetal retardation in the well-fed adolescent sheep discussed earlier.

In contrast to dietary manipulation, a number of studies have examined the effects of administration of porcine somatotrophin/growth hormone (pST/pGH) during gestation on fetal muscle development. Previous research has shown that pST treatment during gestation is capable of affecting placental and/or fetal growth (Rehfeldt et al., 1993; Kelley et al., 1995; Sterle et al., 1995). Administration of pST to pregnant sows during specific periods in early gestation (day 10 to 24) induced the formation of significantly more fibres in fetal ST muscle. This represented a higher growth capacity of skeletal muscle and provided further evidence that critical periods exist in gestation, during which time muscle development in the fetuses may be permanently affected (Rehfeldt et al., 1993). More recently, Rehfeldt et al. (2001) demonstrated that maternal treatment with pST (from day 10 to 27) enhanced the expression of Myf5 and MyoD, and increased total fibre number at birth (primary and secondary fibres), indicating that muscle cell proliferation was prolonged and/or occurred at a higher rate in response to pST treatment. The result was a subsequent increase in formation of secondary muscle fibres. Interestingly, the effects of maternal pST treatment on fetal growth was greatest in the most growth-restricted piglets in each litter. Rehfeldt et al. (2001) discussed possible mechanisms by which pST treatment stimulates embryonic cell proliferation, including increased nutrient availability in maternal blood and in the embryo. A higher nutrient availability may have affected cell proliferation directly or indirectly via hormones or growth factors controlled by nutrient intake.

Gatford et al. (2003) investigated the effects of varying maternal nutrition and maternal treatment with pGH during the second quarter of pregnancy (day 25 to 50) in gilts on semitendinous muscle CSA and fibre composition of the progeny. Higher maternal feed allowance increased the densities of total and secondary muscle fibres and the secondary:primary fibre ratio at 61 days of age. Maternal pGH treatment did not alter fibre densities, but increased the cross-sectional area of the ST muscle, which may be partially explained by increased maternal plasma glucose. Thus maternal nutrition and pGH treatment independently altered muscle characteristics. The results of Gatford et al. (2003) confirmed and extended the previous results of Dwyer et al. (1994), since they observed an increase in the density of secondary fibres in the muscle of progeny that were of median birthweight for their litter (as opposed to the smallest littermates). Furthermore, the effect on muscle fibres was seen with a smaller increase in maternal feed allowance to that used by Dwyer et al. (1994). Gatford et al. (2003) speculated that the mechanisms by which nutrition and pGH treatment during pregnancy prior to secondary fibre development influenced muscle characteristics of postnatal progeny could include improved ability of the placenta to transfer nutrients to the fetus due to IGF-II in the maternal circulation. Increased maternal plasma glucose may partially account for the increase in progeny semitendinosus CSA in response to maternal pGH treatment. A point of interest is that consistent with other reports, increased maternal nutrition (Dwyer et al., 1994) or pGH treatment (Kelley et al., 1995) during the second quarter of pregnancy did not increase weight of progeny at birth, indicating that effects on muscle fibre development may be observed in the absence of an effect on overall birth weight. A very recent study by Gatford et al. (2004) examined the effects of long and short-term treament with pST during pregnancy in underfed pigs. Whilst no effect on birth weight was seen in progeny following 25 days of maternal pST treatment (day 25-50 of gestation), 75 days of pST treatment (day 25-100) resulted in an increase in progeny size at birth. However, muscle fibre characteristics were not studied in this experiment.

Gatford et al. (2003) observed a positive association between maternal body size prior to treatment and total and secondary fibre density in progeny ST muscle at 61 days postnatally, consistent with the degree of maternal constraint of fetal growth (as discussed in section 2.2.2.2), since larger gilts are more likely to have more energy reserves to support fetal growth. In addition, due to low genetic heterogeneity, heavier gilts are probably more mature than lighter gilts and will have slightly less nutrient demand for maternal growth during pregnancy, similar to studies of nutrient partitioning in adolescent sheep (Wallace et al., 1996; section 2.2.2.3).

Whilst the majority of studies have examined the relationships between muscle fibre number, birth weight and postnatal growth, Clelland and Stickland (2001) examined satellite cell populations in large and small siblings in six litters of pigs. Satellite cells from small siblings with fewer muscle fibres proliferated at a significantly higher level than the satellite cells from larger siblings with an increased number of muscle fibres. The authors suggested that satellite cells are primed to react positively to any additional nutrition present in the surrounding environment and thus give a boosted rate of proliferation. However, overall growth rates may not be significantly affected, since the number of muscle fibres, and hence the basic framework for growth, has been impaired earlier in development.

2.3.7 Postnatal muscle development in the pig

Muscle fibres undergo further development in terms of muscle fibre hypertrophy from late gestation through the early postnatal weeks, when the highly organized pattern of muscle fibres seen in the adult pig is established. Harrison et al. (1997) carried out a study to determine the early postnatal pattern of myofibre development and differentiation in a range of functionally distinct muscles in the pig. They demonstrated that although myosin ATPase activity and metabolic properties of porcine myofibres are well developed at birth, they continue to mature postnatally. Myofibres continue to differentiate and hypertrophy postnatally as illustrated by changing myofibre proportions and increasing myofibre cross-sectional areas. This suggests that postnatal muscle development can also be modulated by extrinsic factors.

During the time of postnatal muscle fibre maturation, muscle fibre development may be altered both by energy status and by environmental temperature. Harrison et al. (1996) investigated the role of energy status in postnatal regulation of porcine skeletal muscle development. Littermate animals were kept for 3 to 4 weeks on a high or low energy intake, at thermally neutral or low temperature. In general, myofibre hypertrophy was impaired when energy availability for growth was restricted because of either a low energy intake or a high thermoregulatory demand. Energy restriction reduced muscle fibre hypertrophy in all fibre types, as seen by a reduced CSA of fibres in animals on a low versus a high energy intake. This result contrasts with previous findings in adult humans, in which selective preservation of type I slow twitch fibres occurred during a period of reduced energy availability. Selective preservation is advantageous as a means of energy conservation because the energy expenditure is less in slow twitch fibres than in fast twitch fibres. However, Harrison et al. (1996) suggested that selective preservation of fibre size during undernutrition is used as a means of energy conservation only in fully mature fibres. During early postnatal development, energy conservation occurs by a reduction in the rate of hypertrophy of all fibres.

Changes in energy status also markedly affected myofibre differentiation. In the *rhomboideus* muscle, the proportion of type I fibres was greatly increased in the animal kept at 10°C on low feed intake compared with its littermate kept at 26°C on high feed intake (Harrison et al., 1996). The greater proportion of type I fibres in cold acclimated animals was probably due to the increased contractile activity associated with shivering. Therefore, environmental temperature was shown to exert a muscle specific effect on differentiation of myofibres and hence on the thermogenic function of skeletal muscle. These results demonstrate the plasticity of skeletal muscle differentiation to environmental change during postnatal life.

Lefaucheur et al. (1991) examined the influence of environmental temperature on growth, muscle metabolism and meat quality traits. Pigs were subjected to 12°C or 28°C temperatures from 8 kg live weight to slaughter at approximately 90 kg. For similar growth rates, a 12°C environmental temperature dramatically changed the morphology of the animal and muscle and adipose tissue characteristics, resulting in detrimental effects on meat quality.

Nissen et al. (2004) examined intra-litter variation in postnatal growth performance, meat quality and muscle fibre characteristics when littermates were categorized by carcass weight at an average body weight of 104 kg. Both the number and the growth rate of muscle fibres contributed to the intralitter variation in postnatal growth performance.

2.3.7.1 Evidence for the formation of a third generation of muscle fibres in the pig

In contrast to the pattern of muscle development in small mammals, an additional tertiary generation of myotubes has been demonstrated in various large mammals. Tertiary fibre formation has been shown to occur during late fetal and early postnatal development in the muscles of the pig, sheep and man (Dauncey and Gilmour, 1996 and references therein). Mascarello et al. (1992) and Lefaucheur et al. (1995) demonstrated the existence of a third generation of fibres in the pig. Tertiary myotubes used secondary myotubes as support and may account for a substantial proportion of the final number of fibres produced. The identification of a third generation of postnatally produced fibres is in direct contrast with previous data, suggesting that the total number of myofibres is definitively established by 90 days of gestation in the pig (Ashmore et al., 1973; Wigmore and Stickland, 1983). Lefaucheur et al. (1995) suggested that the presence of a third generation of myofibres could be an important mechanism to explain muscle growth in large animals, although further research is needed to determine the origin and destiny of these tertiary fibres.

2.3.7.2 Postnatal catch-up growth

The subject of catch-up growth has been reviewed by Boersma and Wit (1997). Catch-up growth may be defined as a height velocity growth phase above the statistical limits of normality for age and/or maturity during a defined period of time, following a transient period of growth inhibition. Boersma and Wit (1997) go on to define the difference between catch-up growth and compensatory growth. Although the two terms appear to be synonymous, in contrast to catch-up growth, compensatory growth is used not only to address the growth of the whole organism, but also to describe overgrowth of a single organ or part of an organ when another part is removed (e.g. regeneration of liver tissue after partial hepatectomy). Whilst compensatory growth describes the type of growth that occurs after the loss of an actual mass of tissue and may be viewed as being controlled by a feedback mechanism, catch-up growth is rapid growth that compensates for the loss of potential tissue and cannot be accounted for by a simple feedback mechanism. Different types of catch-up growth have been defined in the human, but are beyond the scope of this chapter. Boersma and Wit (1997) reviewed six factors of importance for the extent of compensatory growth in animal experiments, including the nature, severity and duration of the nutritional insult, in addition to the stage of development at the start of undernutrition, and the relative rate at which a species matures. The pattern of realimentation is also important, as the higher the plane of nutrition upon refeeding, the more rapid and the greater the recovery of weight. In general, the earlier in life the nutritional insult is imposed, the more serious and permanent its effects would be. Since all organs and tissues grow first by hyperplasia and supsequently by hypertrophy, reduction in cell numbers at an early stage in life, results in permanent stunting, whereas reduction in cell size is more likely to be fully recoverable (as discussed by Boersma and Wit, 1997).

Handel and Stickland (1988) investigated the relationship between muscle cellularity and catch-up growth between 23 pairs of low birth weight Large White pigs and their heaviest birth weight littermates. Small littermates (mean birth weight 939g) had on average, lower live weights at slaughter and a lower number of muscle fibres than their large littermates (mean birth weight 2085g). Pigs within the low birth weight category exhibited various degrees of catch-up growth with their heaviest littermates. Relative live weights at slaughter and relative muscle fibre numbers of small and large littermates were determined by expressing the value for the small littermate as a proportion of the corresponding value for its large littermate. Small littermates exhibiting a good degree of catch-up growth (>0.84 relative slaughter weight) and possessed fibre numbers which were not significantly different from those of their large littermates. These results suggested that pigs that exhibit an appreciable degree of catch-up growth always contain high relative numbers of fibres in their muscles. Those which show a poor degree of catch-up growth exhibit a range of fibre numbers, because there may be environmental and other factors which prevent some pigs from realizing their full growth potential. Based on these results, Handel and Stickland advocated muscle fibre number at birth as an indicator of postnatal growth potential.

2.3.8 Muscle fibres as factors for meat quality

In addition to affecting growth performance, several studies have shown that properties of muscle such as number of fibres or fibre type can affect meat quality. Meat quality can be defined as 'the total degree of satisfaction that a meat gives the consumer' (as reviewed by Karlsson et al., 1999), which is an extremely subjective definition. The three major attributes of meat, which affect consumer satisfaction, are tenderness, juiciness and flavour, with tenderness being the most important in determining the overall acceptability of meat. The goals for various muscle quality parameters obviously vary between species, an extreme example being that of tenderness, which should be increased in ruminants or pigs but not in species such as fish. However, the precise factors that determine eating quality remain unknown. Meat quality is currently assessed by measuring biophysical and chemical properties, such as water-holding capacity, colour and light reflectance, pH, pigment content, shear force, intra-muscular fat content and protein extractability (Karlsson et al., 1999). Huff-Lonergan et al. (2002) examined correlations among selected pork quality traits and suggested that changes in some quality traits can affect many other meat quality attributes. An understanding of the relationships among biochemical measurements and sensory and processing characteristics is essential for controlling variation in pork quality.

Generally research has focused on understanding and controlling factors and changes associated with slaughter and subsequent post-mortem events, especially the link between pre-slaughter stress susceptibility or halothane genotype and glycogen depletion and the occurrence of pale, soft and exudative (PSE)-meat (Depreux et al., 2002). However, a number of studies have also linked events occurring during myogenesis with meat quality parameters. Muscle fibre number is an important factor in the production of meat animals. Increased fibre number has been shown to correlate with faster, more efficient growth and the production of animals with improved meat quality. The selection of animals exhibiting high primary fibre number, and nutritional supplementation during gestation to improve secondary fibre formation of offspring, has been suggested as a method to maximize muscle fibre number in pigs (Stickland, 1995).

Traditional pig breeding programs have been based on selection for high growth rate, high feed conversion and high lean meat percentage. The inclusion of meat quality traits into breeding programs is of growing interest. Karlsson et al. (1999) reviewed studies that estimated heritabilities and correlation coefficients between meat quality traits. Muscle fibre traits have been shown to have moderate to high heritabilities in pigs. Larzul et al. (1997) studied phenotypic and genetic correlations of longissimus muscle fibre characteristics in Large White pigs and concluded that these traits have medium heritability and significant genetic correlations with meat and carcass quality characteristics.

Maltin et al. (1997) investigated the specific role of muscle fibre characteristics in accounting for the variation in eating quality. Evaluation of samples from 125 pigs from 8 different breeding companies indicated that the diameter of the fast twitch oxidative glycolytic fibres contributed to variation in texture of meat. Genetic differences were observed in fibre type distribution, implying that certain breeding strategies select either deliberately or inadvertently for characteristics which impact muscle biology and eating quality.

Chang et al. (2003) also examined the relationship between MHC fibre types and meat quality traits between muscles within breeds, and between the same muscles of different breeds (traditional Berkshire and Tamworth and modern Duroc-based and Large White-based breeds). Clear differences in the relative abundance of MHC IIa, IIx and IIb fibres between fast *longissimus dorsi* (LD) and slow *psoas* muscles were observed within breeds. However, there was no clear-cut relationship between the relative abundance of a particular fibre type and a specific meat quality trait, consistent across both muscles and all breeds. The authors concluded that this finding was consistent with the view that a given fibre type can have considerable variations in phenotype between breeds and even between muscles which could result in contrasting correlations with meat quality traits.

Inconsistencies in fibre type classification methods have been blamed for discrepancies in results of studies of the associations between meat quality traits and fibre type characteristics. However, selection for leaner pigs and for a higher proportion of large muscle fibres, especially of type IIB, has been associated with poor capillarisation and consequently an insufficient delivery of oxygen and substrates and elimination of end products such as CO_2 and lactate, resulting in decreased meat quality. Generally a high number of smaller diameter muscle fibres produced meat with a better technological quality (Karlsson et al., 1999).

2.4 Conclusions

The past century has seen important advances in research on placental and fetal growth. However, despite the fact that much effort has already been invested in studying the role of the placenta in the regulation of feto-maternal exchange, there are still questions remaining, particularly with respect to the range of factors that interact to regulate placental and fetal growth and development.

Placental function is critical for normal fetal growth and development. In agriculture, adequate placental function and its effect on fetal mortality and subsequent growth of offspring may have important consequences for maximizing economic returns. Poor growth *in utero* is also associated with a failure to thrive after birth and decreased muscle development. Muscle fibre number is clearly an important determinant of postnatal growth, as demonstrated in cases of nutrient restriction or growth retardation *in utero*. For example, in the pig, littermates with a high fibre number tend to grow faster and more efficiently than littermates with a lower fibre number. Development of muscle fibres can be affected by nutritional status both pre- and postnatally, and by IUGR. Figure 2.10 summarises the various factors influencing prenatal development and, therefore, postnatal outcome. Evidence also indicates that inadequate placental function has subsequent implications for human disease in adult life, through the programming of fetal and thus postnatal control systems. Therefore, the factors affecting placental function and fetal tissue accretion and differentiation *in utero*, have an important role in determining the subsequent health and life expectancy of the animal.

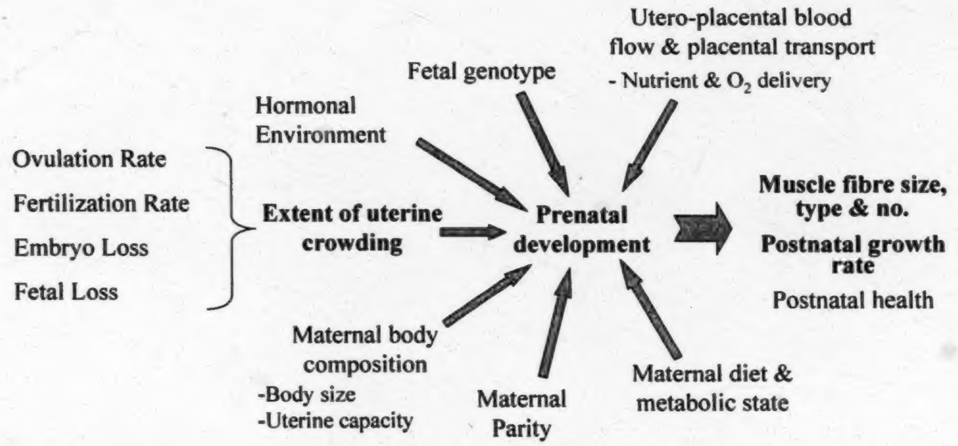

Utero-placental blood flow & placental transport
- Nutrient & O_2 delivery
Fetal genotype
Hormonal Environment
Ovulation Rate
Fertilization Rate
Embryo Loss
Fetal Loss
Extent of uterine crowding
Prenatal development
Muscle fibre size, type & no.
Postnatal growth rate
Postnatal health
Maternal body composition
-Body size
-Uterine capacity
Maternal Parity
Maternal diet & metabolic state

Figure 2.10 Summary diagram representing the various factors influencing prenatal development and therefore postnatal outcome in the pig.

Increased knowledge of the mechanisms by which the pre- and postnatal environment affects fetal survival and development (muscle development in particular) will have significant implications for the agricultural industry. The acquisition of appropriate muscle function is important for the well being of all young mammals and is particularly important in the swine industry for maximizing economic returns in terms of growth rate and meat quality. Based on the observation that the pattern of prenatal loss may vary in existing commercial dam-line sows, the following chapters describe a series of experiments to investigate the extent to which the number of conceptuses *in utero* affects placental and fetal development in the pig.

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CHAPTER THREE

EFFECTS OF MODERATE INTRAUTERINE CROWDING ON FETAL DEVELOPMENT IN COMMERCIAL DAM-LINE GILTS

3.1 INTRODUCTION

Traditionally, pre-implantation losses are thought to be the largest proportion of prenatal loss in the pig (as reviewed by Ashworth and Pickard, 1998); smaller losses post-implantation result in embryo numbers *in utero* that reflect uterine capacity. Results from studies of embryonic survival are generally directed towards development of management protocols that are assumed to improve embryonic survival as a key determinant of litter size born in gilts and weaned first parity sows. However, driven by high ovulation rates in existing commercial dam-line females, (Foxcroft, 1997, Orzechowski, 1998, Vonnahme et al., 2002) the patterns of prenatal loss may be changing with increasing losses during the post-implantation stages of gestation. As a consequence, developing conceptuses are likely subjected to increased uterine crowding.

The components of litter size (ovulation rate, embryonic survival and uterine capacity) responsive to genetic selection are well established (Johnson et al., 1985), with the consensus that selection for both ovulation rate and uterine capacity might be the most productive approach to increase litter size using genetic selection programs (Johnson et al., 1999). The concept of uterine capacity has been widely studied using different animal models to examine effects of crowding *in utero*. Techniques have included uterine ligation, oviduct resection and unilateral hysterectomy and ovariectomy (UHO; Christenson et al., 1987), superovulation, and embryo transfer. Uterine capacity was reported to be a limiting factor for litter size born when the number of embryos exceeds 14 (Dziuk, 1968) and to become a limiting factor for fetal survival after day 25 of gestation (Fenton et al., 1970). The suggestion of Knight et al. (1977) that day 30 to 40 of gestation was the critical period when uterine capacity exerts its effects was supported by subsequent studies in both intact and UHO females (as reviewed by Vallet, 2000). Restricting the length of uterus available to each fetus revealed that 36cm of initial uterine length was required for fetal survival and development (Wu et al., 1989). Whilst these studies have addressed important aspects of uterine capacity, there is a lack of information on the impact of uterine crowding on the development of surviving littermates and the quality, as opposed to the quantity, of offspring produced.

Uterine crowding in early pregnancy detrimentally affects placental development in gilts (Almeida et al., 2000) and higher parity sows (Vonnahme et al., 2002; Chapter 6). In turn, limited placental growth will likely affect fetal development, piglet birth weight and postnatal growth capacity, unless placental efficiency later in gestation compensates for such effects (Biensen et al., 1998). The pathological features of intrauterine growth retardation (IUGR) in swine have been described (Adams, 1971; Dickerson et al., 1971; Widdowson, 1971; Flecknell et al., 1981; Bauer et al., 1998). A common finding is that the brain is the organ least affected by growth retardation, and the brain:liver weight ratio can be used as an effective measure of IUGR (Bauer et al., 1998). Based on earlier findings (Almeida et al., 2000) we hypothesized that even in a proportion of gilts, existing levels of intra-uterine crowding would elicit equivalent "brain-sparing" effects, and that in a situation analogous to IUGR induced by nutritional deprivation of the sow, detrimental effects on critical organ development would be observed. The present study was one of a series of experiments (Chapter 5; Chapter 6) that explored the relationships between levels of uterine crowding, placental size and various aspects of fetal and neonatal development in the pig.

3.2 MATERIALS AND METHODS

3.2.1 Animals

The experiment was conducted at the Swine Research Unit of the University of Alberta, in barns with a controlled environment. Genex Hybrid F1 gilts (Genex Swine Group, Regina, Saskatchewan, Canada; n = 33) were fed as per standard farm protocols. All experimental procedures were carried out in accordance with the guidelines of the

Canadian Council for Animal Care and under authorization from the University of Alberta Animal Policy and Welfare Committee (protocol # 99-33D).

3.2.2 Heat checking, breeding and blood sampling

Starting at day 18 of either the second, third or fourth oestrous cycle, gilts were checked for oestrus every 12 h (0700 and 1900) using the back pressure test during periods of fence line contact with mature vasectomized boars. Gilts were artificially inseminated 12 and 24 h after the onset of standing oestrus with semen (1.5×10^9 spermatozoa/dose) from one of three fertile boars and day of first insemination was designated as day 1 of gestation. A single blood sample was obtained by jugular puncture 72 h after the onset of standing heat, for determination of circulating progesterone concentrations. Signs of a return to oestrus were recorded between days 18 and 22 post-insemination and pregnancy was confirmed at day 25 of gestation using Real Time Ultrasound (RTU).

3.2.3 Ultrasound measurements

3.2.3.1 Preliminary ultrasound studies

A preliminary study was carried out to establish the ultrasound technique. Reproductive tracts were collected from pregnant gilts (n=10) after slaughter between days 25 and 31 of gestation and transuterine ultrasonography carried out (Pie Medical Scanner 200, model 41480, Can Medical, Kingston, Ontario, Canada) using a 5.0 to 7.5 MHz multiple angle transducer to determine embryo number and crown-rump length. Tracts were subsequently dissected and actual crown rump lengths of individual embryos recorded to determine the accuracy of ultrasound measurement.

3.2.3.2 Final surgery ultrasound protocol

Gilts (n=33) underwent ventral midline laparotomy under general anaesthesia at day 30 ± 1 of gestation. Gilts were taken off feed for at least 12 hours before surgery but given free access to fresh water. The animals were restrained with a nose snare, and then given an intravenous short acting general anaesthetic (5% solution of sodium thiopental, 'Pentothal', Merial Ltd, Iselin, New Jersey, USA; dosage: 6.6ml/kg body weight) via an ear vein. Anaesthesia was maintained with a closed circuit system of inhalation general anaesthetic halothane ("fluothane") and oxygen via a facemask and nitrous oxide was also used in combination with halothane for more effective analgesia. Heart rate and respiration rate were monitored by a surgical technician. Anaesthetized gilts were placed in dorsal recumbency on a U shaped table (cradle). Each limb was tied to the table. The surgical site was prepared and draped in accordance with established sterile surgical procedures in veterinary surgery. The surgeons were gowned, gloved and masked.

An incision was made through the abdominal skin on the ventral midline, commencing 2 cm posterior to the umbilicus and extending 10 to 12 cm posteriorly. The subcutaneous fat was blunt dissected to the abdominal aponeurosis (junction of the conjoined abdominal musculature fascia), which was incised. The underlying layer of peritoneal fat and finally the peritoneum were then incised to gain access to the abdominal cavity. The gravid uterus was located and exteriorized in sections. The exposed tissues were kept covered and moist with sterile pads and pre-warmed sterile physiological saline throughout the operation.

Implantation sites were visualized, embryos were located by gentle palpation of the uterus (Figure 3.1a), and the ultrasound probe enclosed in a sterile cover (CIV-Flex general purpose sterile ultrasound transducer cover kit; CIVCO Medical Instruments, Kalona, Iowa, USA) was applied to the wall of the uterus (Figure 3.1b). The number of viable embryos was determined by observing the heartbeat of each embryo once it was located by ultrasound. Each ovary was briefly exposed to record ovulation rate as the number of visible corpora lutea.

A prophylactic antibiotic was administered (either 'Trivetrin', Coopers Agrofarm Inc., Ajax, Ontario, Canada or 'Borgal', Hoechst Roussel Vet Canada Inc., Regina, Saskatchewan, Canada, (both contain Trimethoprim 40mg and Sulfadoxine 200mg); dosage: 2ml into the abdominal cavity).

The peritoneum and musculature were closed in separate layers using absorbable sutures; the peritoneum using continuous cat gut sutures, the abdominal muscle layer (if off the midline), and the subcutaneous layer by interrupted cat gut sutures. The skin was closed with a continuous, absorbable 'vicryl' suture line. The sutured skin incision was sprayed with an antiseptic solution topical wound dressing, ('Boroform', Hoechst Roussel Vet, Warren, New Jersey, USA). The animal was given a prophylactic, broad spectrum, long-acting antibiotic by single dose intramuscular injection ('Liquamycin LA 200' (Oxytetracycline 200mg/ml), Pfizer Animal Health, Exton, Pennsylvania, USA; dosage: 1ml/10kg body weight or alternatively 'Biomycin' (Oxytetracycline 200mg/ml), Boehringer Ingelheim Animal Health Inc., St Joseph Missouri USA; at the same dosage). Any treatments administered were recorded as per quality assurance guidelines.

Post-operatively, the animal was returned to a clean, heated, individual pen with free access to water to allow recovery from anaesthesia. Sows were regularly observed for complications and for evidence of a return to normal food and water intake. After surgery, sows were returned to the gestating sow feeding schedule as per SRU standard operating procedures. Sows were housed at the University of Alberta Metabolic Unit for one week post surgery and were subsequently returned to stall housing at the Swine Research Unit (SRU).

3.2.4 Placental tagging procedure

Gilts were closely supervised throughout farrowing and each piglet was matched to its placenta using the umbilical tagging procedure described by Wilson et al. (1998). Briefly, gilts were induced to farrow by peri-vaginal injection of Planate (Schering Canada, Point Clare, Quebec, Canada) on day 113 ± 1 of gestation. Gilts were then observed hourly for signs of impending parturition. Once milk let down or vulval swelling and lubrication were observed, gilts were monitored continuously until farrowing and placental expulsion was complete. As each piglet was expelled, the umbilical cord was double ligated with two lengths of umbilical tape, each with a matched numbered tag. The umbilical cords were then cut between the tags, allowing the placental end of the cord with its numbered tag to retract into the vagina. Each piglet was numbered, sexed and weighed at birth. After expulsion, placentae were collected and their weights were recorded. The ratio of piglet weight:placental weight was used as a measure of placental efficiency.

3.2.5 Necropsy procedure

With the exception of a sow that only had one live and two stillborn piglets, two representative day-old piglets from each litter were necropsied and organ weights were recorded. To exclude extremes of fetal development, the two piglets chosen had the closest weights to the mean birth weight of the litter. Anaesthesia was induced and maintained using a closed circuit system of the inhalation general anaesthetic, halothane (Fluothane; Ayerst Laboratories, Saint-Laurent, Quebec, Canada), while a heart puncture was performed to obtain a blood sample for measurement of plasma insulin-like growth factor I (IGF-I). Piglets were then killed by an overdose of halothane. Immediately following euthanasia, a midline incision was made to allow removal and weighing of internal organs; heart, lungs, liver, kidneys, adrenal glands, spleen and pancreas. After removal and weighing of the brain, the empty carcass was also weighed. The mean relative piglet organ weights (actual organ weight divided by the actual body weight) were determined to further examine the pattern of organ growth in animals of different birth weights. Semitendinosus muscles were dissected, mounted on aluminium foil and frozen in liquid nitrogen (-196°C). Samples were stored at -80°C until used for electrophoretic analyses of myosin heavy chain (MHC) isoform composition by SDS-PAGE.

3.2.6 Progesterone radioimmunoassay

The gilt plasma samples were analyzed for progesterone concentrations in a single assay using a 'Coat-a-Count' radioimmunoassay kit (DPC, Los Angeles, USA), previously validated for pig plasma (Mao and Foxcroft, 1998). The intra-assay CV was 6.99%, and the sensitivity of the single assay run was 0.1 ng/ml at 84.62% bound.

3.2.7 IGF-I radioimmunoassay

Piglet plasma IGF-I concentrations were determined using the homologous double antibody RIA described by Cosgrove et al. (1992), with modifications relating to the antiserum as described by Novak et al. (2002). All samples were analyzed in duplicate. 100 µl of plasma was initially extracted with 3 ml of acid ethanol. Radio inert recovery was 134% ± 7.94 and samples were not corrected for recovery. The intra-assay CV was 7.02 %, and sensitivity of the single assay run was 24.01 ng/ml at 85.23% bound. Diluted plasma samples showed parallelism to the standard curve.

3.2.8 Myosin extraction

Frozen muscles were pulverized and extracted in six volumes of a buffer containing 0.3 mol/l KCl, 5 mmol/l $MgCl_2$, 5 mmol/l EGTA, 100 mmol/l $Na_4P_2O_7$, 1 mmol/l dithiothreitol and 5 mg/ml Complete Mini Protease Inhibitor (Roche Diagnostics GmBH, Mannheim, Germany), pH 8.5. The solution was stirred for 30 min on ice and cleared by centrifugation for 5 min at 12,000 x g at 4°C. The supernatants were diluted 1:1 (v/v) with glycerol and stored at -20°C. Protein concentrations were determined using the Bradford procedure (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.9 Standard SDS-PAGE

The MHC complement of whole muscle extracts was analysed by SDS-PAGE using a slightly modified version (Putman et al., 2003) of the method described by Hämäläinen and Pette (1996). Briefly, the separating gel contained 7% (w/v) polyacrylamide and 35% (v/v) glycerol, and the stacking gel was composed of 4% (w/v) polyacrylamide and 25% (v/v) glycerol. The upper buffer (25 mmol/l Tris-base, 192 mmol/l glycine, 10 mmol/l SDS) was supplemented with 0.2% (v/v) 2-mercaptoethanol. Before loading, extracts were incubated for 5 min at 100°C in a buffer containing 2.3% (w/v) SDS, 8% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 500 mmol/l Tris-base (pH 7.2), 0.1% (w/v) bromophenol blue and 44% (w/v) sucrose. Samples were cleared by centrifugation at 12,000 x g at 4°C, and 0.75 µg of total protein was loaded in each well. MHC isoforms were separated at 10°C for 24 h at 275 V (constant voltage) and visualized by silver staining. The relative MHC isoform abundance were quantified densitometrically using the Syngene Chemigenius gel documentation system and GeneTools gel analysis software (Syngene, Cambridge, UK). The scheme used for identifying the various MHC isoforms was previously validated by Western blotting (Chapter 6) and by comparison with extracts from rodent muscles.

3.2.10 Statistical analyses

To ensure the independence of observations, sow was used as the experimental unit for analysis, and piglet weights, placental weights from the whole litter and piglet organ weights from the two representative piglets were averaged within each sow before analysis. Relevant associations between ovulation rate, embryo number at day 30, littersize born, average placental weight, average litter weight, placental efficiency, plasma progesterone and piglet organ and birth weights were examined using the Insight procedure of SAS (SAS Institute Inc., Cary, NC). Data from the preliminary ultrasound study were analyzed using the General Linear Model procedure, in a model using day of gestation as the class variable. Ultrasound and actual length measurements were averaged over sows and the insight procedure was used to examine the correlation between values. The Wilcoxen Signed Rank Test from the Univariate procedure of SAS was used as a non-parametric test to examine whether the average difference between the two measurement methods was significant. Significance was considered as $P < 0.05$ and results are presented as means ± SEM.

3.3 RESULTS

3.3.1 Preliminary ultrasound data

In the preliminary ultrasound study, actual crown rump length measurements were affected by day of gestation ($P < 0.001$), as expected. The mean ultrasound length measurement of 2.17 ± 0.07 cm was an underestimate of the mean actual length measurement of 2.46 ± 0.09 cm. Furthermore, there was no correlation between the two measurements ($P = 0.796$) and the average difference between the two measurement methods was significantly different from 0 ($P < 0.001$) using the Wilcoxen Signed Rank Test. The Null hypothesis that there is no difference in the two methods of measurement (actual measurement vs ultrasound measurement) was, therefore, rejected. Although an objective measurement of embryo size was not considered possible based on these preliminary results, ultrasound was still used during laparotomy to get an accurate count of viable embryo number based on the presence of a heartbeat for each embryo. Embryo numbers at day 30 varied from 3 to 18.

3.3.2 General results

Of the 33 gilts that were allocated to the experiment, a total of 23 gilts deemed to be in good health status and with no fertility problems apparent at farrowing were used for the final analysis. From these 23 gilts, 45 necropsied piglets were analyzed. Removals were largely due to a clinical outbreak of porcine circovirus (PCV2) in the University of Alberta Swine Herd, which resulted in abortions, and an increase in stillbirths and fetal mummification in some gilts bred. If the incidence of mummified or stillborn fetuses was higher than that prior to the PCV2 outbreak, gilts were removed from analysis. The production data from this outbreak have been documented (Harding, 2004) and is thought to be one of the few incidences when PCV2 has been involved in overt reproductive failure. In the 23 gilts studied, the number of embryos at day 30 was not associated with ovulation rate ($P = 0.994$) but was strongly related to littersize at term ($R^2 = 0.73$, $P < 0.001$; Figure 3.3).

Although there was a strong positive association between average placental weight at term and average birth weight ($R^2 = 0.76$, $P < 0.001$; Figure 3.4a), neither placental weight nor birth weight showed a strong inverse relationship to litter size at term ($P = 0.11$ and $R^2 = 0.16$, $P = 0.05$, respectively; Figures 3.4b and 3.5), suggesting that uterine capacity had only a moderate effect on intra-uterine development, as determined by fetal weight. Placental efficiency was calculated as the piglet weight:placental weight ratio and there was a strong negative association between placental efficiency and placental weight ($R^2 = 0.57$, $P = < 0.001$). However, there was no relationship between placental efficiency and piglet birth weight ($P = 0.122$), or with litter size at term ($P = 0.894$).

3.3.3 Piglet necropsy data

As piglet birth weights remained unchanged during the PCV2 outbreak (Harding, 2004) necropsy data were deemed to be a reliable estimate of normal development, although the number of embryos at day 30 was probably lower than normal because of the lower embryo survival (Table 3.1). Empty carcass weights were negatively associated with litter size at term ($R^2 = 0.19$, $P = 0.030$). When the relationship between mean body weight and mean absolute weight of various organs was examined, all organ weights showed a positive association with body weight (Table 3.2). However, brain:body weight and adrenal:body weight ratio exhibited the lowest determination coefficients. An inverse relationship was found between the mean relative mass of the neonatal brain (calculated as the mean brain to neonatal body weight ratio) and the mean neonatal body weight ($R^2 = 0.80$, $P < 0.001$; Figure 3.6a). In addition, a significant positive relationship between mean relative spleen weight and mean body weight was observed ($R^2 = 0.23$, $P = 0.02$; Figure 3.6b). This was not the case with other organs, including the liver, heart, lungs, pancreas, kidneys, adrenals and carcass for which no significant relationship was seen between relative organ weight and piglet body weight (Table 3.2). Brain:liver weight ratio was negatively related to mean piglet birth weight ($R^2 = 0.48$, $P < 0.001$; Figure 3.7a) and with mean placental weight ($R^2 = 0.24$, $P = 0.018$; Figure 3.7b), and positively associated with litter size at term ($R^2 = 0.35$, $P = 0.003$; Figure 3.8).

3.3.4 Progesterone assay data

Gilt plasma progesterone concentrations at 72 h after onset of standing heat ranged between 2.9 and 22.4 ng/ml. There was no significant relationship between percentage embryo survival at day 30 and progesterone concentration ($P = 0.231$; Figure 3.9).

3.3.5 IGF-I assay data

Piglet plasma concentrations of IGF-I ranged between 45.9 and 117.5 ng/ml (absolute values). Mean piglet IGF-I concentrations per litter (based on measurements from 2 piglets) showed no relationship to mean piglet birth weight ($P = 0.869$), mean piglet carcass weight (P = 0.920), mean placental weight (P = 0.953) or with brain:liver weight ratio (P = 0.335).

3.3.6 Standard SDS-PAGE

SDS-PAGE results are presented in Table 3.3 as the mean isoform distribution (%) for each of the four myosin isoforms previously identified by Western Blotting (Chapter 6). The embryonic MHC isoform was the most abundant followed by IIa and fetal MHC in similar quantities. Type Iβ MHC was present in the lowest quantity. None of the myosin isoforms were related to litter size, birth weights, placental weights or brain:liver weight ratio (P > 0.1).

3.4 DISCUSSION

The unfortunate occurrence of a clinical outbreak of Porcine Circovirus (PCV2), which affected the gilts used in this study, compromises the value of the data. Even though animals showing signs of the disease at farrowing were omitted from the data set, it is possible that the 23 animals used in final analysis may have been infected at subclinical levels and although they displayed no outward signs of illness, disease could still have affected fetal physiology. Despite the disease status, data were still considered to be useful to examine the relationships between various reproductive parameters, as discussed in the following paragraphs.

The lack of a relationship between embryo numbers at day 30 and ovulation rate in this group of gilts is consistent with results in mixed parity sows (Chapter 4) but in contrast to the results seen by Vonnahme et al. (2002) in multiparous sows. The absence of such a relationship in the present study is likely due to the combination of a relatively low mean ovulation rate (15.6 ± 0.6 compared to 26.6 ± 0.4 in the multiparous sow population studied by Vonnahme and colleagues, 2002) and relatively high embryonic loss (34.8%). The substantial range of embryo survival to day 30 in the current population (21.4 to 100%) removes the expected relationship between ovulation rate and embryo numbers at day 30.

The strong positive relationship between the number of embryos at day 30 of gestation and litter size at term ($R^2 = 0.72$: $P < 0.001$) is not surprising in these animals with the relatively low mean ovulation rate and relatively poor embryonic survival to day 30, post-implantation embryonic loss, due to limitations imposed by uterine capacity was unlikely. Therefore, conceptuses present in the uterus at day 30 of gestation remain until term. In comparison to the present study, the gilts used by Almeida et al. (2000) had a higher mean ovulation rate (17.1 ± 0.6 vs 15.6 ± 0.64), an increased number of live embryos at day 28 (14.3 ± 0.9 vs 10.0 ± 0.76) and higher embryo survival (83.6 ± 4.3 vs 65.2 ± 4.9%).

A strong positive relationship between average placental weight at term and average birth weight ($R^2 = 0.76$, $P < 0.001$; Figure 3.4a) is consistent with data from previous studies (Biensen et al., 1999; Wilson and Ford, 2000). However, compared to data from a previous study by Bauer et al. (1998) and data from our own group (Chapter 6), placental weight was not related to litter size at term ($P = 0.11$; Figure 3.4b). Furthermore, as birth weight showed a significant but weak inverse relationship to litter size at term ($R^2 = 0.16$, $P = 0.05$; Figure 3.5), these results suggest that uterine capacity had only a moderate effect on intrauterine development in these animals, as measured by fetal weight. However, after piglet necropsy and removal of internal organs, a slightly stronger inverse relationship than that observed for birth weight was observed between the empty carcass weights and litter size at term ($R^2 = 0.20$, $P = 0.030$).

The positive associations observed between absolute piglet organ weights and body weight were as expected. The weights of the brain and the adrenal glands exhibited the weakest correlations with body weight. The analysis of relative organ weights (organ:body weight ratios) can be used to express neonatal organ mass as a proportion of body weight, to investigate further the pattern of organ development over the wide range of neonatal body weights observed at farrowing (848 to 1913g). The relationships between relative organ weights and fetal or neonatal body weight, have traditionally been used to illustrate patterns of organ growth in individuals of different body weights. This method is particularly useful to illustrate the concept of "brain sparing", as shown by the significant inverse relationship between the mean relative mass of the neonatal brain and the mean neonatal body weight ($R^2 = 0.73$, $P < 0.001$). The increase in relative brain mass in smaller piglets indicates the maintenance of disproportionate brain growth in smaller piglets. In addition, a significant positive association between mean relative spleen weight and mean piglet body weight was observed ($R^2 = 0.23$, $P = 0.02$), illustrating that in contrast to the brain, the relative spleen mass was increased in larger piglets. These data suggest that larger piglets tend to have disproportionately large spleens compared to piglets of lower body weight. The function of the spleen during fetal life is to produce red blood cells and the data from the current study are consistent with the suggestion put forward by Vallet et al. (2003) that fetal erythropoiesis is impaired under conditions of uterine crowding, although spleen volume was not measured in their study. No significant relationship was seen between neonatal body weight and the relative weights of other organs, including the liver, heart, lungs, pancreas, kidneys, adrenals and carcass, which may have been due to the high variation in organ weight measurements.

McMillen et al. (2001) saw similar effects on relative brain mass in a cohort of normally grown and growth-restricted sheep fetuses, and a similar pattern of adrenal gland development. However, they also saw a direct relationship between the relative mass of fetal liver and fetal body weight, as well as a "threshold effect" in the pattern of fetal kidney growth, whereby growth of the fetal kidney occurred in proportion to body weight until fetal body weight decreased below about 2 kg, at which point kidney mass was then maintained disproportionate to fetal body weight. If such relationships exist in the pig, they were not observed in our data, possibly due to the large variation in organ measurements and the fact that the organ weight measurements used were the mean of two representative piglets per litter.

The brain:liver weight ratio has previously been used as another definitive measure of intra-uterine growth retardation (Bauer et al., 1998). The negative correlation between brain:liver weight ratio and mean piglet birth weight ($R^2 = 0.48$, $P < 0.001$) and mean placental weight ($R^2 = 0.24$, $P = 0.018$), clearly demonstrated the detrimental effects of low birth weight and decreased placental size on neonatal development. Additionally, brain:liver weight ratio was positively correlated with litter size at term ($R^2 = 0.35$, $P = 0.003$). Clearly, brain sparing (as indicated by a high brain:liver weight ratio), occurs to a greater extent in lower birth weight animals. McMillen et al. (2001) have suggested that the maintenance of brain mass appears to be of primary importance for all fetuses, whether they are of normal birth weight or growth-restricted. Therefore, whereas compensatory mechanism may maintain disproportionate brain growth in growth-restricted fetuses, similar physiological mechanisms must operate, albeit to a lesser extent, to ensure that brain mass is maintained within an optimal range even in apparently normally grown animals.

In the current experiment, the significant relationships between littersize at term and brain:liver weight ratio were observed in neonates that had not been subjected to crowding *in utero*. However, even with the relatively "uncrowded" situation observed, at day 30, evidence for the occurrence of brain sparing was seen in the inverse relationship between the mean relative mass of the fetal brain and the mean fetal body weight. The increased brain:liver weight ratio observed both in lower birth weight animals and with larger litter sizes, clearly indicates that the "brain sparing" effect will occur in neonates derived from crowded litters. Results from Chapter 6 have shown that brain sparing is associated with other measures of IUGR, including effects on muscle fibre development. Detrimental effects of uterine crowding on such an economically important aspect of fetal development are a concern for the swine industry and the results of the present experiment indicate that even with a modest degree of uterine crowding, effects on fetal development are observed. This may contribute to variation in postnatal development, which is a major limiting factor for effective all-in/all-out management of grow-finish facilities.

The mean isoform distribution (%) for each of the four myosin isoforms previously identified by western blotting (Chapter 6) was investigated. The embryonic MHC isoform was the most abundant followed by IIa and fetal MHC in similar quantities. Type Iβ MHC was present in the lowest quantity. These are the first data from our group on the distribution of myosin isoforms in neonatal pig semitendinosus muscle and establish the basal conditions for future comparisons of relative MHC isoform distribution. Interestingly, neonatal tissue showed an upregulation of MHC IIa, accompanied by an upregulation of embryonic MHC in comparison to day 90 tissues (Chapter 6), whilst fetal MHC was decreased in neonatal tissue. MHC Iβ levels were similar in both day 90 and neonatal tissue. The increase in embryonic MHC observed in neonatal tissue may be a function of adaptive myogenesis in response to the stresses associated with weight bearing and stretch within the first day of postnatal life. Putman et al. (1998) examined the changes in satellite cell content and myosin isoforms in low frequency-stimulated fast muscle of rats. Similar results were observed, whereby an upregulation in developmental isoforms was accompanied by an upregulation of MHC IIa. Whilst species differences undoubtedly exist between the pig and the rat, and chronic low frequency stimulation is used as a protocol for studying adaptive responses to maximum endurance training (fast to slow fibre type transitions) rather than the limited feeding and exploratory activity of newborn piglets, the mechanisms that regulate MHC isoform expression must be the same. Putman et al. (1998) suggested that the combined expression of MHC IIa and MHC developmental isoforms may be due to the tandem organization of the two corresponding genes, alternatively the recruitment of satellite cells are required for the addition of new nuclei to growing fibres and consequently MHC embryonic is expressed through the normal course of satellite cell terminal differentiation. Whilst research on porcine fetal muscle development has been carried out by several groups (Dwyer et al., 1994; Lefaucheur et al., 1995; Fazarinc et al., 1995; Lefaucheur et al., 2002), further investigations are clearly required to confirm the patterns of MHC isoform distribution in developing pig muscle. None of the myosin isoforms were correlated with litter size, birth weights, placental weights or brain:liver weight ratio ($P > 0.1$), consistent with the findings described in later chapters.

Placental efficiency is calculated by the fetal/neonatal weight:placental weight ratio (Wilson et al., 1999). A lack of association between placental efficiency and litter size, and a strong negative correlation between placental efficiency and placental weight

($R^2 = 0.57$, $P = < 0.001$), suggests that piglet birth weight is more dependent on placental size, than placental efficiency. These data are consistent with the hypothesis (Biensen et al., 1998; Wilson et al., 1998) that during late gestation, breed-specific mechanisms exist, which maintain optimal fetal growth. Meishan fetal growth depends on an increase in placental vascular density whereas York (white-line) fetuses rely on an increased surface area available for placental exchange. However, as suggested by Vallet et al. (2002), the fetal weight:placental weight ratio may not be a good measure of placental efficiency in intact gilts where littersize is low. Over the wide range of fetal and placental weights observed in 121 fetuses at 105 days of gestation, a curvilinear relationship was found between these two variables and placental and fetal weights were not related once placental weights exceeded 200g. Based on these findings, the authors proposed that given a large enough placenta to meet the needs of the fetus, fetal weight is no longer controlled by placental transport but by factors intrinsic to the fetus.

Gilt plasma progesterone concentrations at 72h after onset of standing heat ranged between 2.9 and 22.4 ng/ml, which is consistent with plasma progesterone concentrations measured in previous studies (Pharazyn et al., 1991; Almeida et al., 2000). Although no correlation was observed between embryo survival and progesterone concentrations in the present study, variation in embryo survival appeared to decrease as progesterone concentration increased, consistent with the suggestion that increasing progesterone concentrations in early pregnancy may support better embryo survival by providing a more appropriate uterine environment (Foxcroft, 1997).

The mitogenic peptides IGF-I and -II mediate tissue growth and differentiation in a variety of cell types and circulate in plasma in association with IGF binding proteins (IGFBPs). Their effects are mediated by binding to specific type 1 and 2 IGF receptors on the surface of target cells. Lee et al. (1991, 1993) characterized the ontogeny of circulating IGFs and IGFBPs during fetal and early postnatal development in the pig and suggested that IGF-I may be primarily a postnatal growth factor in pigs. Lee et al. (1991) found serum IGF-I levels to increase during the latter half of fetal life from 11 ± 1 ng/ml on day 60 to 37 ± 3 ng/ml on day 112 and further increased to 227 ± 22 ng/ml on day 42 of postnatal life. These values are in a similar range to those observed in the current study (45.9 to 117.5 ng/ml). Lee et al. (1993) suggested that in young pigs, skeletal muscle may be a significant source of circulating IGF-I, since muscle represents the bulk of total body weight and IGF-I mRNAs and type 1 receptors are expressed at relatively high and low levels, respectively, in hindlimb muscles. Piglet plasma concentrations of IGF-I in the current study, showed no relationship to mean piglet birth weight (P = 0.87), carcass weight (P = 0.92), placental weight (P = 0.953) or brain:liver weight ratio (P = 0.335), unlike the surprising inverse correlation observed by Bauer et al. (1998) between plasma IGF-I and birth weight. Conversely, plasma IGF-I concentrations observed in the current study are an order of magnitude lower than the results obtained by Bauer and coworkers. However, the three-fold range of values in the present study was similar to the range of values obtained by Bauer et al. (1998). Bauer and coworkers did not speculate on the cause of the significant inverse relationship that they observed. Our data are also in contrast to the data of Wise et al. (1997), who observed an increase in day 104 gestation fetal serum IGF-I concentrations with increasing fetal weight. However, this study considered fetuses from 28 litters that showed extremes in fetal weight within a litter (250 to 1350g) and the fetuses selected for analysis represented the extremes in fetal weights within these litters. Clearly there are contradictory results regarding the relationships between fetal IGF-1 concentrations and body weight. Unfortunately, the results of the current study do not provide any evidence to further elucidate the link that IGF-1 may have with fetal birth weight or IUGR.

In conclusion, despite possible PCV2 disease-related limitations on reproductive performance, even moderate crowding of the uterus in the early period of gestation affected fetal development of the surviving conceptuses in a manner analogous to IUGR, in the absence of effects on birth weight. These data raise important questions for fetal and postnatal development, and parallel studies (Chapter 6) suggest that the development of fetal muscle fibres will be affected. Given that an improvement in embryonic survival or increases in ovulation rates have the potential to produce even greater numbers of conceptuses at day 30 of gestation in gilts (Almeida et al., 2000) and sows (Chapter 6), and knowing that this has negative effects for early placental development, variation in subsequent fetal development *in utero* could be a potential cause of deficiencies in postnatal growth performance.

Table 3.1 Reproductive characteristics of gilts (n=23); means ± SEM.

Parameter	Means ± SEM	Range
Ovulation rate	15.6 ± 0.6	12-28
Number of embryos at day 30	10.0 ± 0.8	3-18
Embryo survival rate[a], %	65.2 ± 4.9	21-100
Embryo loss to day 30[b], %	34.8	0-79
Litter size born[c]	8.5 ± 0.7	3-15
Fetal wt, g	1338 ± 49	848-1913
Placental wt, g	289.2 ± 14.6	189-478
Placental efficiency[d]	4.8 ±0.15	3.9-6.8
Fetal plasma IGF-I concentration, ng/ml	64.3 ± 2.8	47 – 99

[a] Embryo survival rate is defined as number of viable fetuses at day 30 of gestation divided by the ovulation rate.

[b] Embryo loss at day 30 is calculated as 100%-Embryo survival rate.

[c] Litter size includes live born piglets and full term stillborns.

[d] Placental efficiency is the fetal weight:placental weight ratio.

Table 3.2 Linear regression analysis of absolute organ weight/body weight and relative organ weight/body weight for each of 23 litters (averages). (Body weights were used as the independent variable x and the various organ weights (absolute or relative) as the dependent variable y. Relative organ weights were calculated as the organ weight/body weight. R^2 represents the coefficient of determination. *All regressions between organ weights and body weights were significant $P < 0.05$).

	Absolute organ weight/body weight			Relative organ weight/body weight		
Organs	Equation	R^2	P	Equation	R^2	P
Brain	$y = 23.4387 + 0.0055\,x$	0.38	0.0019^*	$y = 0.0408 - 1.3\text{x}10^{-5}\,x$	0.80	$<0.0001^*$
Liver	$y = -0.4859 + 0.0323\,x$	0.62	$<0.0001^*$	$y = 0.0366 - 8.2\text{x}10^{-7}\,x$	0.00	0.89
Heart	$y = -1.4867 + 0.0080\,x$	0.66	$<0.0001^*$	$y = 0.0064 + 4.0\text{x}10^{-7}\,x$	0.00	0.68
Lung	$y = -2.2066 + 0.0170\,x$	0.80	$<0.0001^*$	$y = 0.0145 + 7.0\text{x}10^{-7}\,x$	0.01	0.60
Pancreas	$y = -0.3015 + 0.0016\,x$	0.60	$<0.0001^*$	$y = 0.0009 + 3.2\text{x}10^{-7}\,x$	0.08	0.19
Spleen	$y = -0.8340 + 0.0018\,x$	0.66	$<0.0001^*$	$y = 0.0005 + 4.5\text{x}10^{-7}\,x$	0.23	0.02^*
Kidneys	$y = -2.3675 + 0.0096\,x$	0.79	$<0.0001^*$	$y = 0.0065 + 9.9\text{x}10^{-7}\,x$	0.08	0.21
Adrenal	$y = -0.1703 + 0.003\,x$	0.23	0.0264^*	$y = 8.3\text{x}10^{-5} + 8.9\text{x}10^{-8}\,x$	0.05	0.34
Carcass	$y = -9.6301 + 0.7508\,x$	0.98	$<0.0001^*$	$y = 0.7338 + 5.8\text{x}10^{-7}\,x$	0.01	0.72

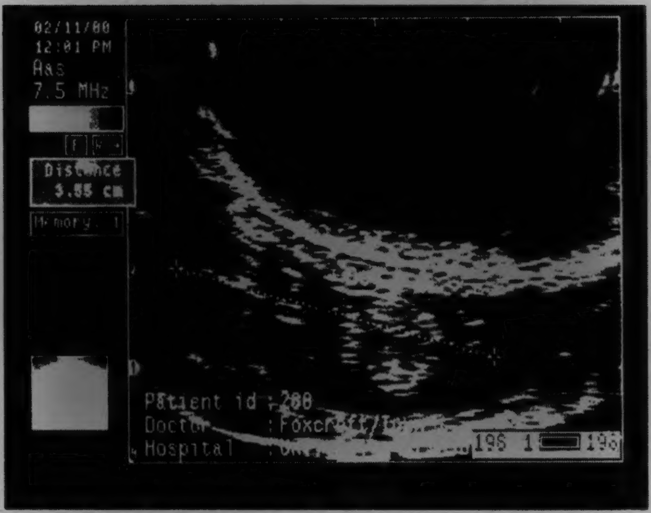
Table 3.3 Relative Myosin Heavy Chain Isoform distribution (mean % ± SEM) in neonatal semitendinosus muscle (N = 23 gilts; n = 45 piglets).

	Semitendinosus Muscle Tissue
Parameter	**Neonatal**
Embryonic MHC (%)	38.35 ± 1.59
Fetal MHC (%)	26.29 ± 1.36
Type IIa MHC (%)	27.03 ± 1.33
Type Iβ MHC (%)	8.33 ± 1.12
Fetal:Adult MHC isoforms	47.74 ± 1.02

Figure 3.1 a) Embryo location by gentle palpation of the uterus and application of the ultrasound probe to the wall of the uterus during midline laparotomy surgery. b) Ultrasound image of a day 30 embryo taken by transuterine ultrasound during midline laparotomy surgery. Note: Crown-Rump-Length (CRL) measurement of 3.55cm taken by ultrasound measurement is likely to be an overestimate of the actual CRL.

a)

b)



Distance 3.55 cm

Memory : 1

Patient id : 200
Doctor : Foxcr [illegible]
Hospital : [illegible]

Figure 3.2 Placental tagging procedure; a) Tagging the umbilical cord. b) Recovery of tagged placentae. c) Piglet identification.

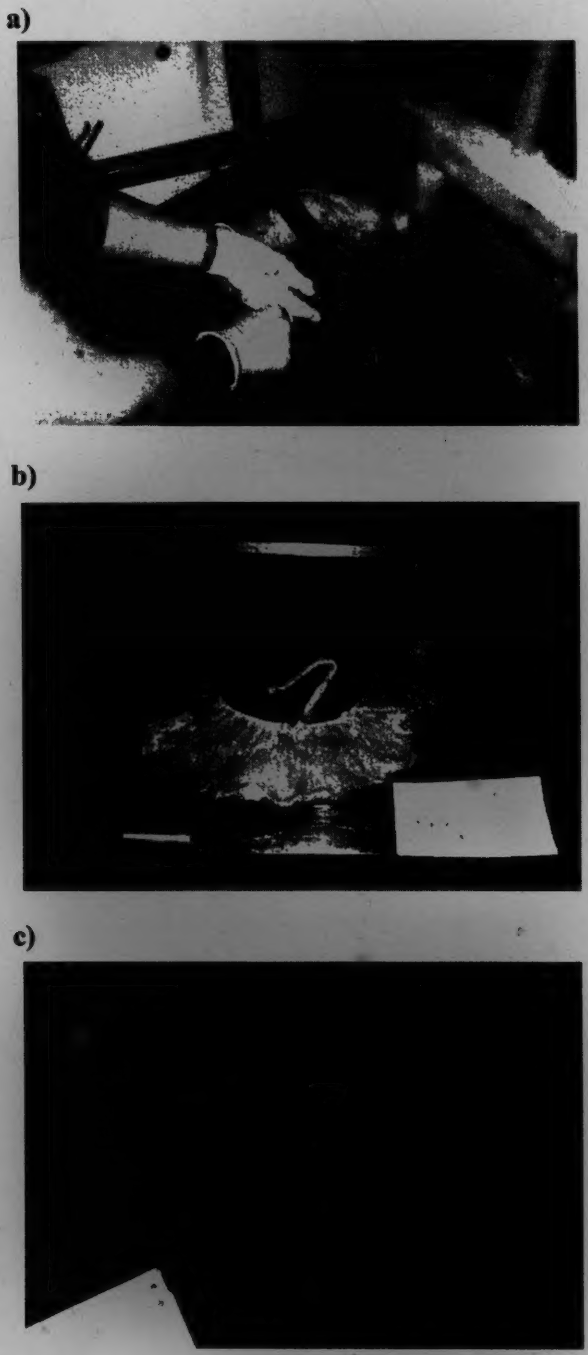


Figure 3.3 Relationship between litter size born and number of embryos at day 30 of gestation (Litter size born = 0.52 + 0.80(Number of embryos), $R^2 = 0.73$, $P < 0.001$). n = 23 gilts.

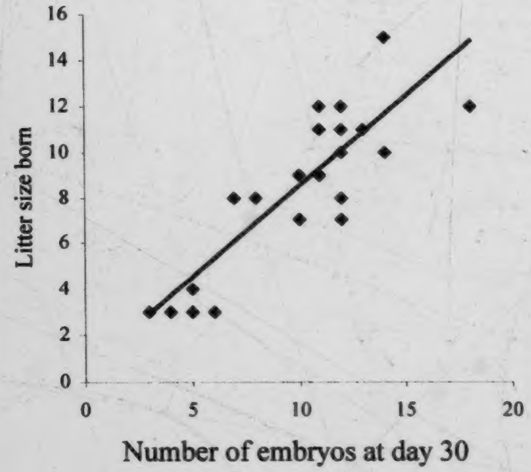


Figure 3.4 Relationship between average placental weight at term and a) average birth weight (Average placental weight = -62.5 + 0.26(Average birth weight), $R^2 = 0.76$, $P < 0.001$) and b) litter size at term ($R^2 = -0.12$, $P = 0.12$). n = 23 gilts.

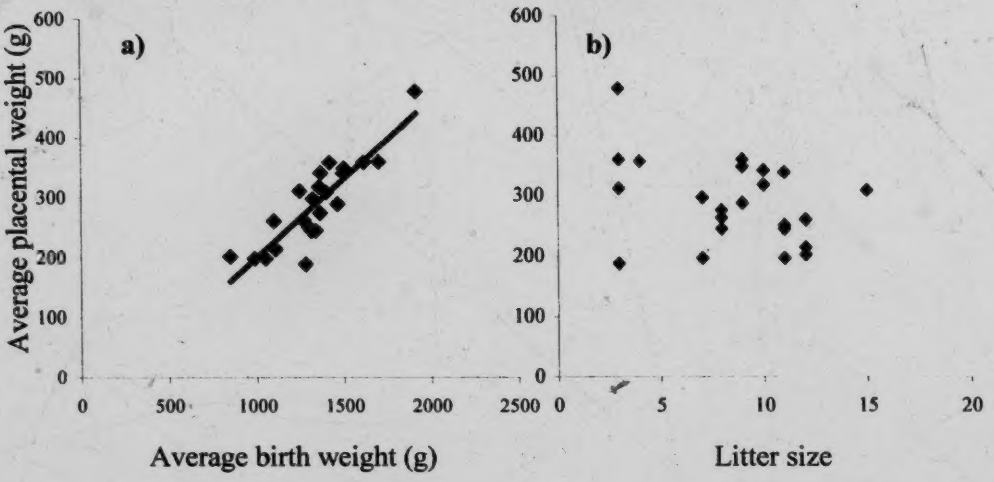


Figure 3.5 Relationship between average birth weight and litter size at term (Average birth weight = 1575.5 – 27.92(Litter size at term), $R^2 = 0.16$, $P < 0.05$) n = 23 gilts.

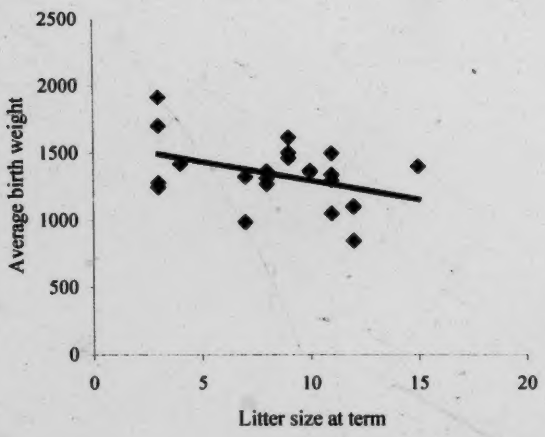


Figure 3.6 a) Relationship between mean relative brain weight (calculated as the mean brain to neonatal body weight ratio) and neonatal body weight (g) (relative brain weight = $9 \times 10^{-9}(\text{body weight})^2 - 4 \times 10^{-5}(\text{body weight}) + 0.058$, $R^2 = 0.80$, $P < 0.0001$) and b) relationship between mean relative spleen weight (calculated as the mean spleen to neonatal body weight ratio) and neonatal body weight (g) (relative spleen weight = $0.0005 + 4.5 \times 10^{-7}(\text{body weight})$, $R^2 = 0.23$, $P = 0.0213$).

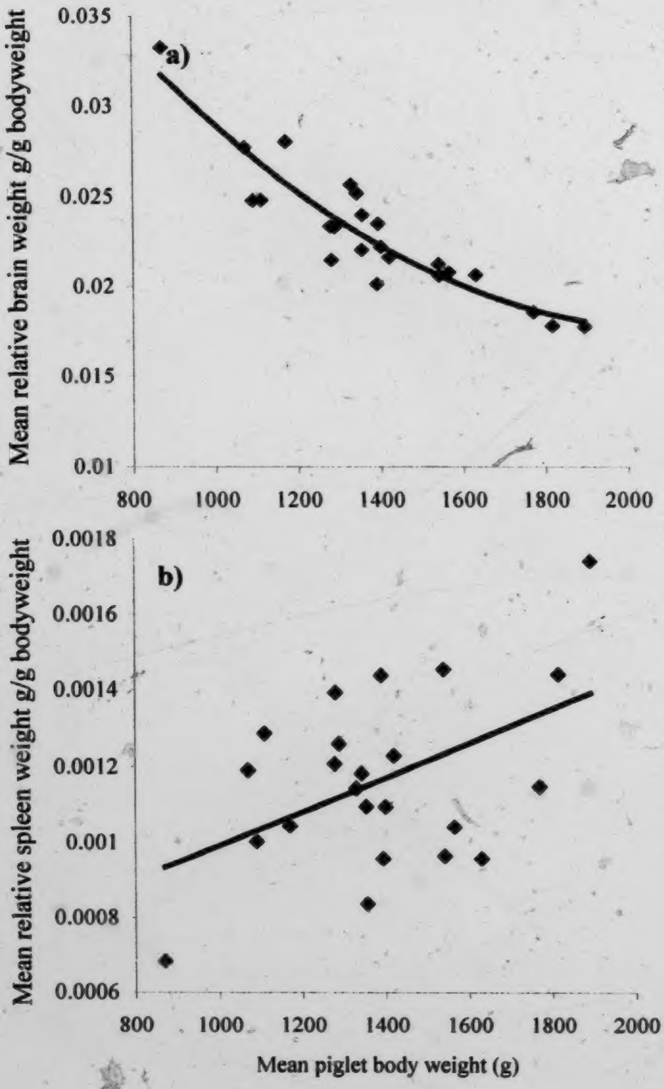


Figure 3.7 Relationship between average brain:liver weight ratio and a) average birth weight (brain:liver weight ratio = 1.37 – 0.0005(average birth weight), R^2 = -0.48, P < 0.001) and b) average placental weight (brain:liver weight ratio = 1.06 – 0.0011 (average placental weight), R^2 = 0.24, P = 0.018). n = 23 gilts.

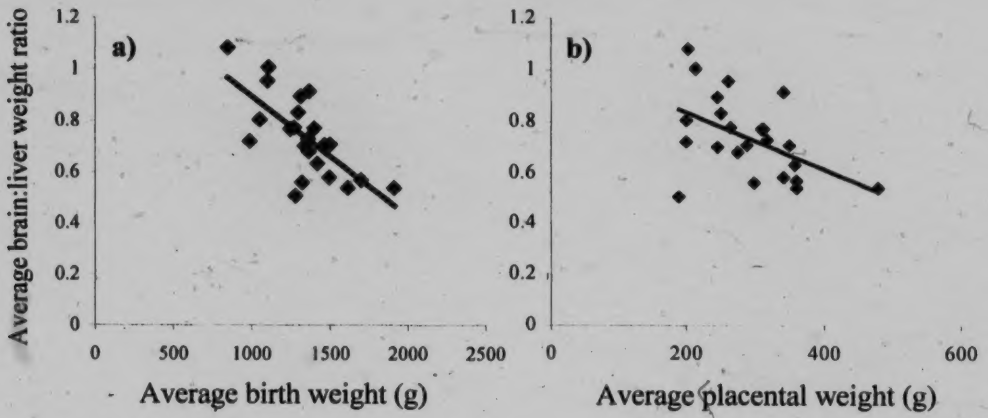


Figure 3.8 Relationship between mean brain:liver weight ratio and litter size at term (brain:liver weight ratio = 0.51 + 0.0273(Litter size), $R^2 = 0.35$, $P = 0.003$). n = 23 gilts.

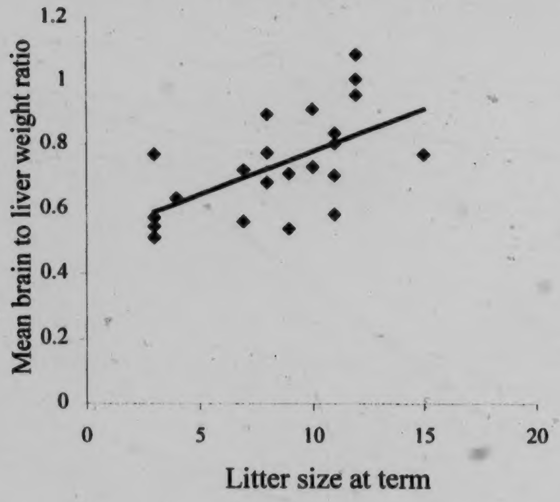
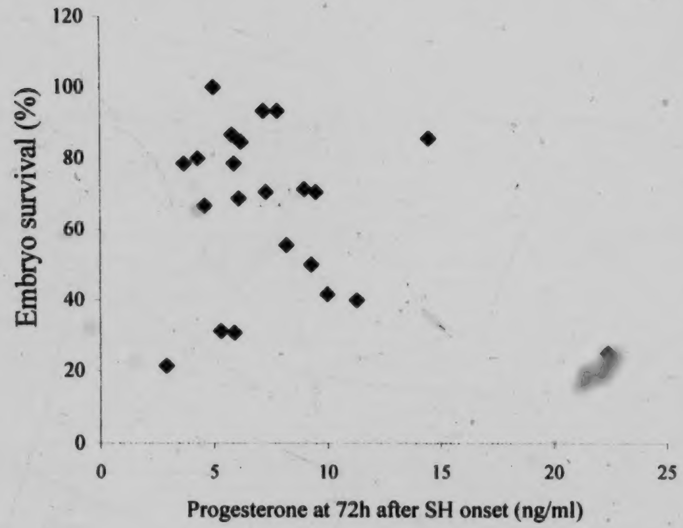


Figure 3.9 Non significant relationship between embryo survival rate at day 30 of gestation and gilt plasma progesterone concentration (ng/ml) at 72h after onset of Standing Heat (P = 0.231).



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CHAPTER FOUR

EMBRYONIC AND FETAL DEVELOPMENT IN A COMMERCIAL DAM-LINE GENOTYPE*

4.1 INTRODUCTION

The concept of uterine capacity as the ultimate constraint on litter size in swine has been widely studied using different animal models to examine effects of crowding *in utero*. Techniques including uterine ligation, oviduct resection, unilateral hysterectomy and ovariectomy (UHO; Christenson, 1987), superovulation, and embryo transfer have been employed and led to the conclusion that when the number of embryos exceeded 14, intrauterine crowding was a limiting factor for litter size born (Dziuk, 1968). Fenton et al. (1970) determined that uterine capacity only becomes a limiting factor for fetal survival after day 25 of gestation and Knight et al. (1977) further defined day 30 to 40 of gestation as the critical period when uterine capacity exerts its effects. Subsequent studies in both intact and UHO females support this conclusion (see Vallet, 2000). Wu et al. (1989) restricted the length of uterus available to each fetus and concluded that 36cm of initial uterine length was required for fetal survival and development. Whilst these early studies addressed uterine capacity in terms of number of embryos, uterine space requirement for embryo survival and time of embryonic loss, less focus has been directed toward the quality of offspring produced. In addition, ovulation rates of animals used in these early studies were relatively low (10 to 12) compared with ovulation rates greater than 25 reported in contemporary commercial sow populations (Vonnahme et al., 2002) and the concept of uterine capacity needs to be re-evaluated in such populations.

The extremes of intrauterine growth retardation (IUGR) or "runting" have been described in the pig (Adams, 1971; Widdowson, 1971; Cooper et al., 1978; Hegarty and

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* A paper based on Chapter 4 by S.C. Town, J.L. Patterson, C. Z. Pereira, G. Gourley, and G.R. Foxcroft has been accepted subject to revision by Animal Reproduction Science.

Allen, 1978; Flecknell et al., 1981), and were identified within a discrete sub-population of lighter weight fetuses (Royston et al., 1982; Wooton et al., 1983). However, conclusions based only on a consideration of fetal weight may overlook critical effects on fetal development that are established early in gestation. Indeed, Hegarty and Allan (1978) reported that within a litter, runts have a reduced muscle growth potential and, as a consequence, needed 23 days longer to reach a weight of approximately 105kg (slaughter weight). In addition, data from a study by Aberle (1984) indicated that naturally occurring IUGR may delay myofibre differentiation, preferentially affecting secondary muscle fibre development.

High ovulation rates associated with only modest increases in litter size born in commercial dam-line sows may result in a dramatic change in the dynamics of prenatal loss, associated with crowding of embryos *in utero* in the immediate post-implantation period and a peak of prenatal loss between day 30 and 50 of gestation (Foxcroft, 1997; Vonnahme et al., 2002). The consequences of this pattern of prenatal loss for placental and fetal development, and as a factor contributing to increased variability in postnatal growth performance, need to be determined. A recent study of associations among within-litter variation in birth weight, and pre-weaning survival and weight gain, also led to the conclusion that selection for increased litter size that results in more low-birth-weight piglets may not be beneficial, unless measures are taken to improve the survival of the low-birth-weight offspring (Milligan et al., 2002). Thus, both the developmental competence of the pigs born, as well as the size of the litter, are critical factors.

Even in gilts with a mean ovulation rate of 17.1 ± 0.6, a negative relationship between the number of conceptuses and placental volume, and a significant positive relationship between placental area and embryo size at day 28 of gestation has been reported (Almeida et al., 2000), suggesting that even moderate uterine crowding in early gestation affects placental and embryonic development. If this is not fully compensated by increased placental efficiency in surviving conceptuses later in gestation, there may be major consequences for prenatal development and postnatal growth performance, analogous to IUGR previously reported in the pig (Widdowson, 1971).

In an initial herd depopulation study (Vonnahme et al., 2002) it was established that mean ovulation rate in a similar population of sows to those used in the present study was 26.6, and that ovulation rate was positively correlated with embryo numbers, and negatively correlated with placental weight, at day 25 of gestation, we wished to further explore possible implications for fetal development. The depopulation of a second commercial sow-breeding unit within Swine Graphics Enterprises provided this opportunity.

The first objective of this study was to extend information on the reproductive characteristics of a large population of contemporary commercial dam-line sows. The second objective was to extend earlier observations (Vonnahme et al., 2002) and determine associations between placental and fetal development in later gestation and parity dependent changes in reproductive efficiency.

4.2 MATERIALS AND METHODS

4.2.1 Animals

In an internal, Porcine Reproductive and Respiratory Syndrome (PRRS) naïve, genetic multiplication program (Swine Graphics Enterprises, Inc., Webster City, Iowa, USA), Pig Improvement Company (PIC-USA, Franklin, Kentucky, USA) Camborough sows were crossed with PIC-derived boars to produce the equivalent of PIC line 1055 F1 gilts for commercial production. These gilts were then mated with crossbred PIC terminal-line 337 boars at production level. Bred females from one such commercial breeding unit (ADL 1 Swine Unit, Osceola, Iowa, USA) were made available for this study as part of a herd depopulation exercise linked to mortality in nursery pigs from the sow unit due to PRRS. Productivity of these sows had previously been adversely affected by infection with two different strains of PRRS virus. However, PRRS virulence within the breeding herd was stabilised by vaccinations with autogenous PRRS vaccines in July and early December 2000, at least 60 days before any of the sows used in the present study were bred.

Weaned sows were bred by AI according to normal herd practice. Females were inseminated each morning that standing oestrus was detected in the presence of a boar, using pooled semen containing approximately 4 billion sperm per AI dose. As part of the study design and in addition to parous sows already bred at the start of the herd depopulation program, groups of gilts were also specifically bred to provide parity 0 data not available previously (Vonnahme et al. 2002). Bred females were checked daily for return to oestrus in the presence of a boar until day 50 of gestation, unless they were allocated to an earlier slaughter group, in which case they were checked for oestrus until the day they were shipped. For the purposes of this study, 454 presumed pregnant females were initially allocated to slaughter on one of three days on the basis of parity, using herd records and individual sow identification. The parity distribution of animals shipped, gilts and first parity (parity 0-1), second and third parity (parity 2-3), and 4th parity and greater (parity 4+) is shown in Figure 4.1. Of the approximately equal numbers of sows initially allocated to be at different stages of gestation at the time of slaughter, data were obtained from the reproductive tracts of 103 of 114 animals at day 20-30, 149 of 163 animals at day 50-55 and 169 of 177 animals at day 85-90 of gestation (total of 421 pregnant tracts at slaughter). Mishandling and loss of tissue during dissection inevitably resulted in some loss of material; the numbers of tracts contributing data to different reproductive characteristics within different parity groups are shown in Table 4.1. Logistical constraints of measuring reproductive tract data from such a large number of animals over a period of only 3 days prevented the identification of individual sows after slaughter, however, the three parity groups were slaughtered on separate days. Given this constraint, the rationale behind the parity group allocations was that in gilts and first parity sows (parity 0-1) ovulation rate and embryonic survival rate would typically be limiting factors for litter size born. In contrast, in second and third parity sows (parity 2-3) reproductive performance would be optimal and uterine capacity, rather than ovulation rate and embryo survival rate, was expected to be the critical constraint on litter size. Sows of fourth parity and greater (parity 4+) were expected to represent sows in which reproductive performance would be declining, associated with increased variability in the uniformity of pigs born in terms of numbers and development.

4.2.2 Slaughter and dissection procedures

Reproductive tracts were dissected at cooperating commercial abattoirs immediately after slaughter (PorkKing Packing Company, Marengo, Illinois; Abbyland, Curtis, Wisconsin). Unfortunately time and manpower constraints at time of slaughter prevented collection of embryo data from day 20-30 parity 0-1 animals, which resulted in a missing cell of data for some reproductive characteristics. However, ovulation rate and number of viable conceptuses were recorded for all other females (Table 4.1, Figures 4.2, 4.3 and 4.4). As discussed in Section 4.4, to avoid extremes of embryonic and placental development associated with conceptuses developing at the oviductal and cervical extremities of the uterus, two conceptuses were dissected from each mid-uterine horn on day 20-30 and embryonic and placental weights recorded. Again, to avoid extremes of fetal development determined by position *in utero*, one representative fetus was selected from each mid uterine horn on day 85-90 of gestation (n=166), and fetal and placental weights were recorded. The fetuses were then dissected to determine brain and liver weights. The brain:liver weight ratio was then used as an estimate of proportional changes in organ development, indicative of the occurrence of IUGR.

4.2.3 Statistical Analysis

To determine the effects of parity group, gestation day at slaughter and their interaction on ovulation rate, embryonic survival rate, number of viable embryos, placental and fetal weights, placental efficiency and fetal brain:liver weight ratio, data were analyzed as appropriate for a completely randomized design. Sow was used as the experimental unit for analysis, and fetal weights, placental weights and organ weights were averaged within each reproductive tract (sow) before analysis. As logistical constraints prevented collection of embryo data from day 20-30 parity 0-1 animals, different statistical models were used to analyse parameters of interest and thus extract the most information from the data available.

Ovulation rate data were complete for all 9 groups of animals, and therefore, were analyzed as a 3 x 3 factorial for parity group and gestational day using the General Linear

Model (GLM) procedure of the Statistical Analysis System (SAS, 1990, SAS Inst. Inc., Cary, NC).

Due to missing embryo data from the day 20-30, parity 0-1 group (see Table 1), embryo survival rate and the number of live embryos were first analyzed as a 3 x 2 factorial (using only data from parity 2-3 and parity 4+ sows, at all gestational days, to primarily examine the effect of gestational day). Data were then analyzed as a 2 x 3 factorial using only gestational days 50-55 and 85-90, but all three parity groups, to primarily examine the effect of parity.

Since placental and fetal data were only obtained from day 20-30 animals and 85-90 animals, the effect of parity on conceptus development at these time points was further examined by analyzing placental and embryonic/fetal weights both as a 2 x 2 factorial using data from gestational days 20-30 and 85-90 and parity groups 2-3 and 4+, and using data for all three parities and gestational day 85-90. The effect of parity on brain:liver weight ratio, was also examined using data for all three parities and gestational day 85-90.

Trends analysis (Steel et al., 1997) was used to determine if there was a linear relationship between gestation day and both embryonic/fetal survival rate and the number of viable embryos/fetuses. The Tukey-Kramer test (SAS, 1990) was used for making pairwise comparisons between least squares means.

Relevant associations within gestational age between ovulation rate, number of viable embryos, fetal weight, placental weight, placental efficiency (calculated as the fetal:placental weight ratio; day 20-30 and 85-90), and fetal brain and liver weights and brain:liver weight ratio (day 85-90), were examined using the INSIGHT procedure (SAS, 1990), and only involved animals for which complete data sets were available.

4.3 RESULTS

4.3.1 General results

Of the 454 presumed pregnant animals initially allocated for slaughter, 7 sows died during transport, resulting in 447 presumed pregnant sows slaughtered. Of these, 421 were confirmed pregnant by the presence of viable conceptuses. Conceptuses were classified as viable using semi-objective criteria based on visual appearance of the embryo or fetus and the placenta. In general, if the placenta was well vascularized and did not look necrotic, it was classified as viable. As specified later, data from other sows were excluded from the analysis as a result of damage during tract removal and collection (missing ovaries, etc). Ovulation rate (22.7 ± 0.2 overall), was affected by parity ($P < 0.0001$; Table 4.1) and was higher in parity 2-3 (23.6 ± 0.4) and parity 4+ (24.7 ± 0.4) sows than in parity 0-1 sows (20.2 ± 0.5). As shown in Figure 4.2, approximately 18% of the higher parity sows had ovulation rates ≥ 30. There was no effect of gestational day, nor a parity by gestational day interaction, on ovulation rate.

Analysis of number of viable embryos over all three gestation time points, but using only parity groups 2-3 and 4+ (3 x 2 factorial design), revealed an effect of gestational day ($P < 0.0001$), and a parity by gestational day interaction ($P = 0.04$; Figure 4.3). Within parity groups, there was a negative linear relationship between the mean number of viable embryos/fetuses and gestational day at slaughter in parity 2-3 (mean number of embryos/fetuses = 16.38 - 1.37(gestational day at slaughter); $R^2 = 0.99$; $P = 0.003$) and parity 4+ (mean number of embryos/fetuses = 16.92 - 2.55(gestational day at slaughter); $R^2 = 0.86$; $P < 0.0001$) sows. When analyzed as a 2 x 3 factorial using only gestational days 50-55 and 85-90 but all three parity groups, the number of viable fetuses was affected by parity ($P < 0.0001$; Figure 4.4a) and was higher ($P < 0.05$) in the parity 2-3 sows than in the other parity groups. There was no relationship between ovulation rate and number of live embryos at day 20-30 ($P = 0.18$; Figure 4.2) or day 85-90 ($P = 0.72$).

Survival rate, calculated by dividing the number of viable embryos/fetuses per sow by ovulation rate, was analyzed over all three gestation time points using parity groups 2-3 and 4+ (3 x 2 factorial). Survival was affected by day of gestation ($P < 0.0001$; Table 4.1) and there was a negative linear relationship between survival rate and gestational day (survival rate = 66.7 - 6.6(gestational day at slaughter); $R^2 = 0.83$; $P < 0.0001$). There was no day of gestation by parity interaction for survival rate. The 2 x 3 factorial analysis of embryo survival rate (gestational day 50-55 and 85-90 only, analyzed over all three parities) indicated an effect of parity ($P < 0.0001$; Figure 4.4b) and there was also a negative linear relationship between survival rate and parity ($y = 71.3 - 9.0\ x$; $R^2 = 0.92$; $P < 0.0001$). Again, no gestational day by parity interaction was present.

4.3.2 Fetal necropsy data

Mean embryonic and placental weights from the four day 20-30 conceptuses dissected per sow were available for 79 of the 80 sows slaughtered. The analysis of placental and embryonic/fetal weights, as either a 2 x 2 factorial using data from gestation day 20-30 and 85-90 and parity groups 2-3 and 4+, or using data for all 3 parities and gestation day 85-90, revealed effects of gestation day, and a parity by gestational day interaction, for placental weight (Table 4.1). When the parity by gestational day interaction was examined, the average placental weight for parity 4+ (3.42 ± 0.43g) was less than half that of parity 2-3 (7.55 ± 0.40g) at day 20-30 ($P < 0.0001$). However, at day 85-90, the average placental weight of parity group 2-3 (209.5 ± 8.5g) was lower than both parity 0-1 (235.7 ± 7.3g) and parity 4+ (235.4 ± 7.1g) ($P = 0.05$) which were not different from each other. As expected, there was a significant effect of gestational day on fetal weight, but there was no main effect of parity, or a parity by gestational day interaction.

Average embryonic weight was positively related to average placental weight at day 20-30 of gestation ($R^2 = 0.20$; $P < 0.0001$; Figure 4.5a), but neither average placental weight ($P = 0.75$), nor average embryonic weight ($P = 0.88$), was correlated with number of viable embryos. Data from the two conceptuses dissected per sow were available from 166 of the 175 pregnant sows slaughtered at day 85-90. Average fetal weight was positively associated with average placental weight ($R^2 = 0.29$; $P < 0.0001$; Figure 4.5b) and there was a tendency towards a relationship between average placental weight and number of viable fetuses ($P = 0.057$). However, average fetal weight ($P = 0.32$), was not associated with number of viable fetuses.

Placental efficiency was calculated as the embryonic weight: placental weight ratio. The analysis of placental efficiency (2 x 2 factorial for gestational day 20-30 and 85-90 and parity groups 2-3 and 4+), revealed effects of gestational day, parity, and a parity by gestational day interaction (Table 4.1). When the parity by gestational day interaction was examined, placental efficiency for parity 4+ (0.12 ± 0.01) was seen to be almost double that of parity 2-3 (0.07 ± 0.01) at day 20-30 ($P < 0.0001$), consistent with the placental weight results. The analysis of placental efficiency for all three parities and gestation day 85-90 revealed an effect of parity. At day 85-90, the average placental efficiency of parity group 2-3 (3.68 ± 0.11) was higher ($P < 0.03$) than both parity 0-1 (3.30 ± 0.09) and parity 4+ (3.23 ± 0.09), which were not different ($P > 0.05$).

At day 20-30, placental efficiency was weakly associated with average fetal weight ($R^2 = 0.09$; $P = 0.009$) but was not related to number of viable embryos ($P = 0.34$). However, placental efficiency showed a stronger negative correlation with average placental weight ($R^2 = 0.37$; $P < 0.0001$). Average placental weight at day 85-90 was also negatively correlated to placental efficiency ($R^2 = 0.38$; $P < 0.0001$), whilst average fetal weight showed a very weak positive correlation with placental efficiency ($R^2 = 0.08$; $P = 0.0002$). Placental efficiency was not related to the number of viable fetuses at day 85-90 of gestation ($P = 0.18$).

Parity affected brain:liver weight ratio ($P = 0.01$; Table 4.1), such that parity 0-1 had a higher brain:liver weight ratio than parity 2-3. Brain:liver weight ratio was not different between parity 0-1 and parity 4+, nor between parity 2-3 and parity 4+. Both mean absolute liver weight ($R^2 = 0.\ 56$; $P < 0.0001$; Figure 4.6a) and brain weight ($R^2 = 0.23$; $P < 0.0001$; Figure 4.6b) were positively correlated to average fetal weight. However, when relative organ weights were calculated as the absolute organ weight:body weight ratio, mean relative liver weight was not related to average fetal weight ($P = 0.45$; Figure 4.6c), whilst mean relative brain weight showed a strong negative correlation to mean fetal weight ($R^2 = 0.61$, $P < 0.0001$; Figure 4.6d). The mean brain:liver weight ratio was negatively correlated with mean fetal weight ($R^2 = 0.35$; $P < 0.0001$; Figure 4.7a) and mean placental weight ($R^2 = 0.14$; $P < 0.0001$; Figure 4.7b). However, the mean brain:liver weight ratio was not related to number of viable fetuses ($P = 0.08$).

4.4 DISCUSSION

A previous depopulation exercise with sows of the same genetic lines provided an initial opportunity to establish reproductive characteristics in a large population of contemporary commercial dam-line sows (Vonnahme et al., 2002). In that study, results were obtained from a total of 244 sows of parities 2 to 14, slaughtered on days 25, 36 or 44 of gestation. The present study provided a second opportunity to examine reproductive characteristics in the same population over a greater range of parities (gilts up to parity 16) and over a longer duration of gestation (day 25 to 90). We were then able to interpret the collective data from the two studies against our working hypothesis that high ovulation rates in higher parity sows in contemporary dam-lines could be the driver of critical changes in the dynamics of prenatal loss and hence fetal development. As seen previously in parous sows (Vonnahme et al., 2002), when high ovulation rates are initially associated with even moderate embryonic survival, this results in uterine crowding at day 30 of gestation, and a negative relationship between placental size and embryo number around day 25-30 of gestation. In turn, crowding of embryos *in utero* around day 30 of gestation drove a peak of post-implantation loss between day 30 and 50 of gestation (Vonnahme et al., 2002). Unless placental compensation occurs during and after this time, reduced placental size will have negative consequences for fetal development of the remaining conceptuses and for postnatal outcomes. In many respects, we consider that these effects of *in utero* crowding in swine may be analogous to the effects of IUGR reported both in swine (Bauer et al., 1998; Flecknell et al., 1981; Widdowson, 1971) and in other species (McMillen et al., 2001; Gluckman and Harding, 1997).

The justification for dissecting fetuses for measurement from the mid-uterine horn was to ensure a representative measure of overall effects on fetal development. Wise et al. (1997) studied the relationships of light and heavy fetuses to uterine position and found no differences between fetal weight and uterine position at day 30. However, at day 70 and 104, heavier fetuses were found to be located at the tubal ends, whilst lighter fetuses were found at the cervical ends of the uterus. Since our aim was to evaluate the overall effects of fetal number on the development of the whole litter, we chose to examine the average, rather than the extremes, of fetal development within a litter.

The overall ovulation rate for the present study of 22.7 ± 0.2 is a little lower than the ovulation rate of 26.6 ± 0.4 observed by Vonnahme and colleagues (2002). In part, this resulted from the inclusion of gilts and parity 1 sows that had lower ovulation rates (20.2) than parity 2 and 3 (23.6) or parity 4+ (24.7) sows. Nevertheless, the upper range of ovulations rates recorded, with approximately 50% of higher parity sows having ovulation rates of 25 or higher, and 18% of sows having ovulation rates of 30 or higher, confirms the great disparity between ovulation rate and litter size born in this commercial dam line. Therefore, the collective results of work with the present genotype in addition to the earlier data of Orzechowski (1998) confirm that high ovulation rates in some commercial sow populations have the potential to dramatically affect the pattern of prenatal loss and hence fetal development.

The logistical constraints at time of slaughter, which prevented collection of embryo data from day 20-30 parity 0-1 animals, resulting in a missing cell of data, was clearly very disappointing. However, the opportunity to collect additional data from such a large number of animals from a commercial swine herd, was still considered invaluable. The statistical analyses used were appropriate for the limitations in data collection yet maximised the information obtained.

The extent to which the number of conceptuses at day 30 exceeds uterine capacity to support subsequent fetal development is critically dependent on the interaction between ovulation rate and early embryonic survival. The significant positive relationship between ovulation rate and numbers of viable conceptuses *in utero* at day 25 of gestation established by Vonnahme et al. (2002) was not evident in the present study, and the reason for this is not clear. Embryonic survival to day 20-30 for the parity 2-3 and 4+ groups was only 61.8% in the present study, but is comparable to the 60.2% survival to day 25 observed by Vonnahme et al. (2002). In both studies, this probably reflects the relatively poor, but stable health status of the herds at the time of depopulation. The inclusion of gilts and parity 1 sows in the present study may be partly responsible for the absence of a relationship between ovulation rate and number of viable conceptuses at day 25, as the higher ovulation rates in higher parity sows will tend to exaggerate the degree of crowding *in utero*, and only this population of sows was studied by Vonnahme et al. (2002). Clearly, however, the high embryonic loss to day 20-30 of gestation meant that excessive crowding of embryos *in utero* at day 20-30 was not universally present in the sows included in the present study. Notwithstanding the absence of extreme crowding in this group of animals, important and previously unavailable information on placental function and fetal development was obtained.

The second factor driving a dynamic change in the pattern of prenatal loss would be a level of uterine crowding at day 30 that substantially exceeds uterine capacity later in gestation, such that the peak of prenatal loss would now occur in the immediate post-implantation, rather than in the pre-implantation period. Although *in utero* crowding of embryos was not recorded in the immediate post-implantation period, a significant linear decrease was observed in the number of viable conceptuses in both parity groups examined, decreasing from 15.0 embryos present at day 20-30 in both parity groups to 12.2 fetuses in parity 2-3, and 9.9 fetuses in parity 4+, sows at day 85-90 of gestation. Although no parity effect was observed by Vonnahme et al. (2002), a significant decrease in conceptus number was observed between day 25 and 36 of gestation in that study. Consistent with the effect on number of viable conceptuses, day of gestation affected embryonic/fetal survival, which decreased from 61.8% at day 20-30 to 50.2% at day 50-55 and to 48.7% by day 85-90. Together with estimates of survival from the study by Vonnahme and colleagues (2002) of 60.2% on day 25, 50.1% on day 36 and 46.3% on day 44, these results support the suggestion that the majority of postimplantation loss already occurs by day 50. In both studies, therefore, there was evidence that the number of viable conceptuses at day 25-30 of gestation still exceeded uterine capacity. If the high ovulation rates in these sows were associated with the higher (85-100%) levels of pre-implantation survival reported in studies with high health status commercial dam-line sows (Zak et al., 1997), excessive *in utero* crowding of embryos would be present around day 30 of gestation, and would drive a major peak of prenatal loss before day 50 of gestation. The consequences of this changing dynamic of prenatal loss then becomes a critical question.

The significant effect of parity on the number of conceptuses *in utero*, and the interaction between parity and gestational day on prenatal loss established in the present study, are of interest. The number of fetuses at day 85-90 increased from 11.81 in parity 0-1 animals to 12.24 in parity 2-3 sows and then decreased to 9.86 fetuses in the parity 4+ group. The increased number of viable embryos in parity group 2-3 may be due to the improved uterine environment and improved placental efficiency in these more mature sows, particularly given the equivalent fetal weights observed in this group but a significantly lower placental weight. In contrast, despite maximal ovulation rates and comparable levels of pre-implantation survival in the parity 4+ sows, it appears that the quality of the uterine environment and placental efficiency declines and hence reduces the functional "capacity" of the uterus. In all parities, however, ovulation rate is never a limiting factor for potential litter size born.

Both the negative correlation between allantochorionic volume and number of embryos at day 28 of gestation observed in a study with commercial dam-line gilts by Almeida et al. (2000), and the negative relationship ($r = -0.36$ $P < 0.005$) established by Vonnahme et al. (2002) between the number of conceptuses at day 25 and placental weight in similar sows to those used in the present study, support our contention that high ovulation rates produce detrimental effects on conceptus development as early as day 30 of gestation. In the present study, placental weight was positively associated with embryonic weight at day 20-30 and with fetal weight at day 85-90 of gestation, suggesting a functional relationship between these two variables. Larger piglets are also attached to larger placentae at term (Biensen et al., 1999; Wilson and Ford, 2000; Town et al, 2002). However, neither placental weights nor fetal weights were significantly related to number of viable embryos at day 20-30 in the present study. Given the lack of a negative relationship between ovulation rate and the number of conceptuses at day 20-30 discussed above, this is probably expected, and the lack of an effect may again be related to the particular population of sows studied. As we would expect, neither placental weight nor fetal weights were associated with number of viable fetuses at day 85-90, reflecting a pattern of prenatal loss that limits the number of surviving fetuses to the uterine capacity by the later stages of gestation.

The significant parity by gestational day interaction for placental weight merits consideration. Although placental weight at day 20-30 for parity 4+ sows was less than half that seen in parity 2-3, the lower number of surviving fetuses appears to allow for placental compensation during later gestation, resulting in equal weight fetuses in the Parity 4+ sows, attached to significantly larger, but less efficient, placentae. Consistent with the results of Vonnahme et al. (2002), placental efficiency showed little relationship to embryonic weight at day 20-30 and with fetal weight at day 85-90, but did show a strong negative association with placental weight at day 20-30 and at day 85-90. These results support the suggestion of Vonnahme et al. (2002) that heritable differences in placental efficiency, largely driven by variation in placental size, may be a key determinant of prenatal development and survival in the pig.

The observed positive associations between absolute liver and brain weights and average fetal weight were expected, although the lower coefficient of determination seen with the brain compared to the liver, suggests that brain weight is less dependent on fetal weight and that a "brain-sparing" effect was present. As discussed in Chapter 3, relative organ weights (organ:body weight ratio) can be used to further investigate effects of IUGR on the pattern of organ development over the wide range of fetal body weights observed. This method allows the identification of disproportionate changes of relative organ size with change of absolute body size, which occurs to the greatest extent in the brain.

When relative organ weights are related to fetal weight, the brain sparing effect is more apparent. Relative liver weight showed no relationship to fetal weight, whilst relative brain weight showed a strong negative association with fetal weight. These results confirm data from other studies (Town et al., 2002) in which an increase in relative brain mass with decreasing fetal weight is assumed to reflect a brain sparing effect. In the context of the present study, it is important to recognize that the maintenance of disproportionate brain growth in growth restricted fetuses is occurring even in situations where excessive *in utero* crowding of developing fetuses is not present.

The brain:liver weight ratio was calculated for each fetus as another indicator of growth retardation (Bauer et al., 1998). The mean brain:liver weight ratio was negatively associated with mean fetal weight and mean placental weight as previously observed in neonatal piglets (Town et al., 2002). These relationships demonstrate the detrimental effects of lower fetal and placental weights on organ development. This raises the question about negative consequences for other more commercially important aspects of pre- and postnatal development, such as myogenensis. Although brain:liver weight ratio was not related to number of viable fetuses, a positive trend was observed (P = 0.08). Furthermore, a significant positive association between brain:liver weight ratio in one day old piglets and litter size at term was recently observed in a study of the relationship between embryo survival to day 30 of gestation and subsequent fetal development in gilts (Town et al., 2002). The demonstration of brain sparing effects in situations in which the level of crowding *in utero* in the early post-implantation period is relatively low, compared to the level of crowding that is possible in higher parity sows with ovulation rates of 25 and greater and improved embryonic survival, supports our contention that the changing dynamics of prenatal loss in commercial dam-line sows may have important consequences for the developmental potential of the offspring born. The same mechanisms may also underlie reported detrimental effects of low birth weight in gilts born in large litters (Deligeorgis et al., 1985; Jorgensen, 1989). The results of the data accumulated from our studies of commercial culled sows has, therefore, encouraged us to develop appropriate experimental paradigms for studying direct effects of the pattern of prenatal loss on muscle fiber development.

Our results confirm that high ovulation rates are a characteristic of some contemporary dam-line sows. In the absence of the high embryonic loss seen in the present study, high ovulation rates provide the basis for excessive *in utero* crowding of conceptuses in the early post-implantation period, and a radical shift in the dynamics of prenatal loss. Negative effects of increased numbers of conceptuses *in utero* on placental development around day 30 of gestation reported in our earlier studies, may then have lasting effects on fetal development. Evidence reported here for measurable brain sparing effects in smaller fetuses, even in the absence of serious uterine crowding in early gestation, suggests that such developmental effects are likely. Possible long-term effects on economically important traits like postnatal growth performance suggest that the reproductive characteristics of commercial dam-line sows merit further study.

Table 4.1 Reproductive characteristics of sows (Least Square Means ± SEM).

	Parity group		
Parameter	**0 – 1**	**2 - 3**	**4+**
Ovulation rate (N = 405)	20.18 ± 0.49^{a} (n = 138)	23.59 ± 0.43^{b} (n = 121)	24.71 ± 0.38^{b} (n = 146)
Number of viable conceptuses	N/A	13.65 ± 0.39^{a}	11.81 ± 0.34^{b}
D20-30 Sows (N = 80)	**n = 0**	**n = 37**	**n = 43**
Placental weight (g) at d20-30	Not Available	7.55 ± 0.43^{a}	3.42 ± 0.40^{b}
Fetal weight (g) at d20-30	Not Available	0.46 ± 0.03^{a}	0.40 ± 0.03^{a}
Placental efficiency at d20-30	Not Available	0.07 ± 0.01^{a}	0.12 ± 0.01^{b}
D50-55 Sows (N = 149)	**n = 71**	**n = 31**	**n = 47**
D85-90 Sows (N = 166)	**n = 57**	**n = 48**	**n = 61**
Placental weight (g) at d85-90	235.7 ± 7.3^{a}	209.5 ± 8.5^{b}	235.4 ± 7.1^{a}
Fetal weight (g) at d85-90	751.8 ± 19.4^{a}	755.8 ± 22.3^{a}	738.7 ± 18.7^{a}
Placental efficiency at d85-90	3.30 ± 0.09^{a}	3.68 ± 0.11^{b}	3.23 ± 0.09^{a}
Brain:liver weight ratio at d85-90	1.00 ± 0.03^{a}	0.85 ± 0.04^{b}	0.92 ± 0.03^{ab}
		Gestation day	
Parameter	**20-30 (n = 80)**	**50-55 (n = 74)**	**85-90 (n = 100)**
Number of viable conceptuses	14.98 ± 0.46^{a}	12.17 ± 0.48^{bc}	11.05 ± 0.41^{bc}
Survival rate (%) parities 2-3 and 4+ (N = 254)	61.8 ± 2.1^{a}	50.2 ± 2.2^{b}	48.7 ± 1.9^{b}

LS Means ± SEM within a row with different superscripts differ ($P \leq 0.05$)
"Not Available" denotes data not available for analysis

Figure 4.1 Parity distribution of sows allocated for slaughter (N = 454). Light grey boxes denote parity group 0-1 (n = 147), mid grey boxes denote parity group 2-3 (n = 143) and dark grey boxes denote parity group 4+ (n = 164).

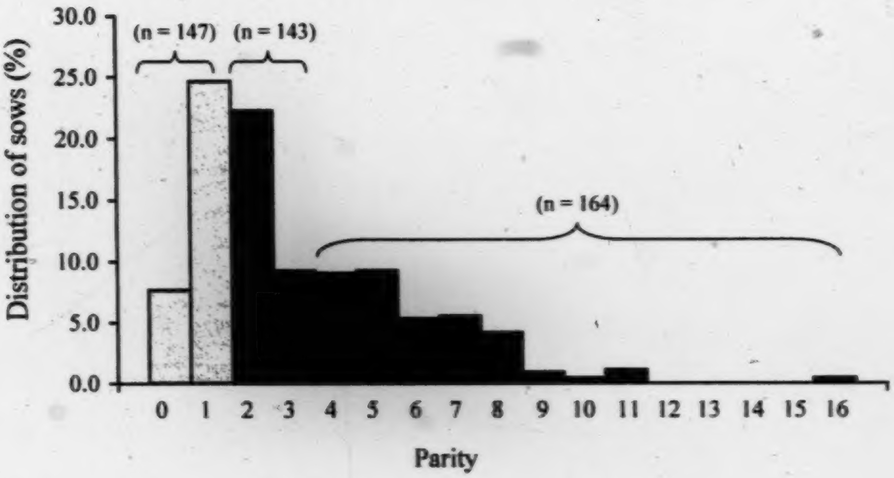


Figure 4.2 Lack of a relationship between ovulation rate and number of viable embryos at day 20-30 of gestation (P = 0.18) in parity groups 2-3 and 4+ (n = 73).

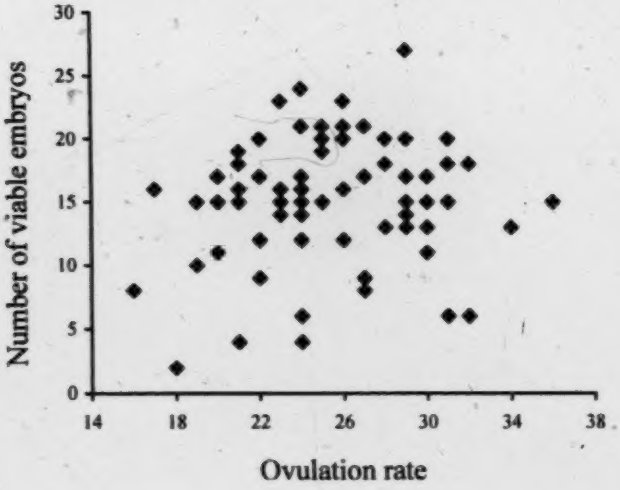


Figure 4.3 3 x 2 factorial analysis of number of viable embryos over all three gestational days for parity groups 2-3 (light bars) and 4+ (dark bars). LS means with different superscripts differ within parity group.

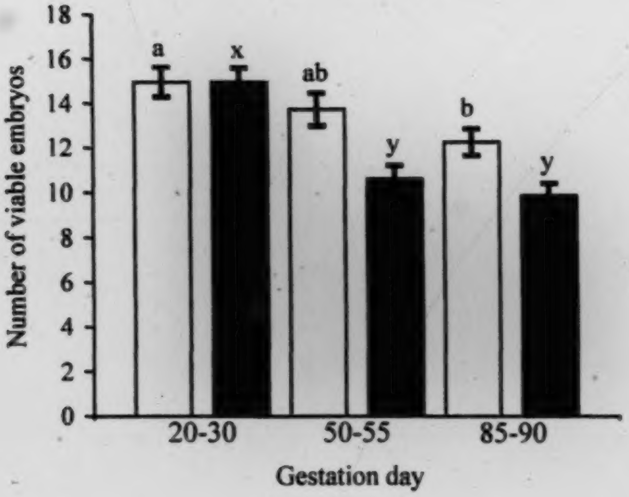


Figure 4.4 Results of 2 x 3 factorial analysis of (a) the number of viable fetuses and (b) % embryonic survival on day 50-55 and 85-90 of gestation using all three parity groups. LSmeans with different superscripts differ. Error bars denote standard error of LS means. Bars that do not share a common letter indicate significantly different values ($P < 0.05$).

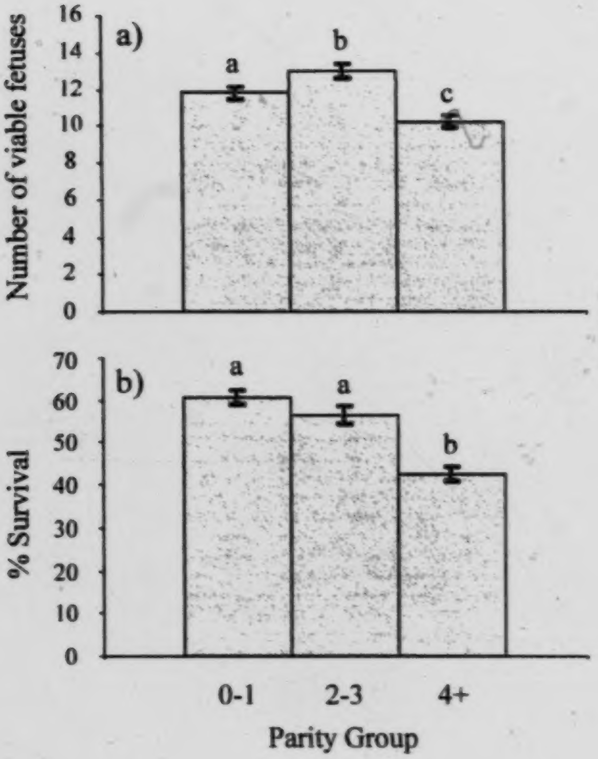


Figure 4.5 Relationship between average placental weight and a) average embryo weight at day 20-30 of gestation (average placental weight = 1.90 + 7.94(average embryonic weight), $R^2 = 0.20$, $P < 0.0001$; n = 79) and b) average fetal weight at day 85-90 of gestation (average placental weight = 71.89 + 0.21(average fetal weight), $R^2 = 0.29$, $P < 0.0001$; n = 166).

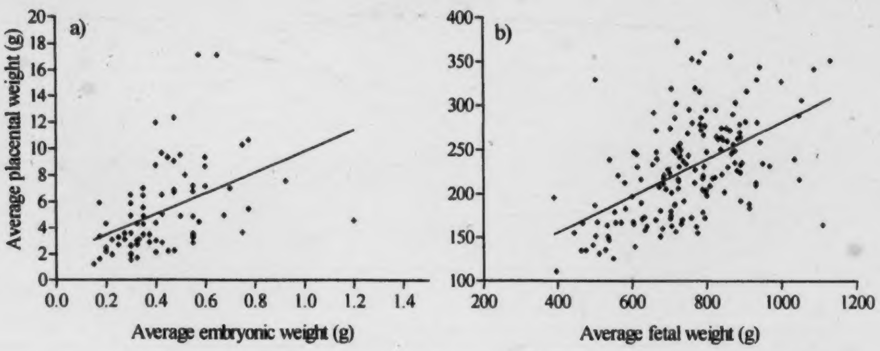


Figure 4.6 Positive relationship between a) average liver weight (liver wt = -1.19 + 0.035(fetal wt), $R^2 = 0.56$, $P < 0.0001$) and b) average brain weight (brain wt = 15.23 + 0.008(fetal wt), $R^2 = 0.23$, $P < 0.0001$), and average fetal weight at day 85-90 of gestation: Non significant relationship ($P = 0.45$) between mean relative liver weight (c) and a negative correlation (relative brain wt = $0.054 - 3.2\text{x}10^{-5}$(fetal wt), $R^2 = 0.61$, $P < 0.0001$) between mean relative brain weight (d) and average fetal weight at day 85-90 of gestation. N = 166.

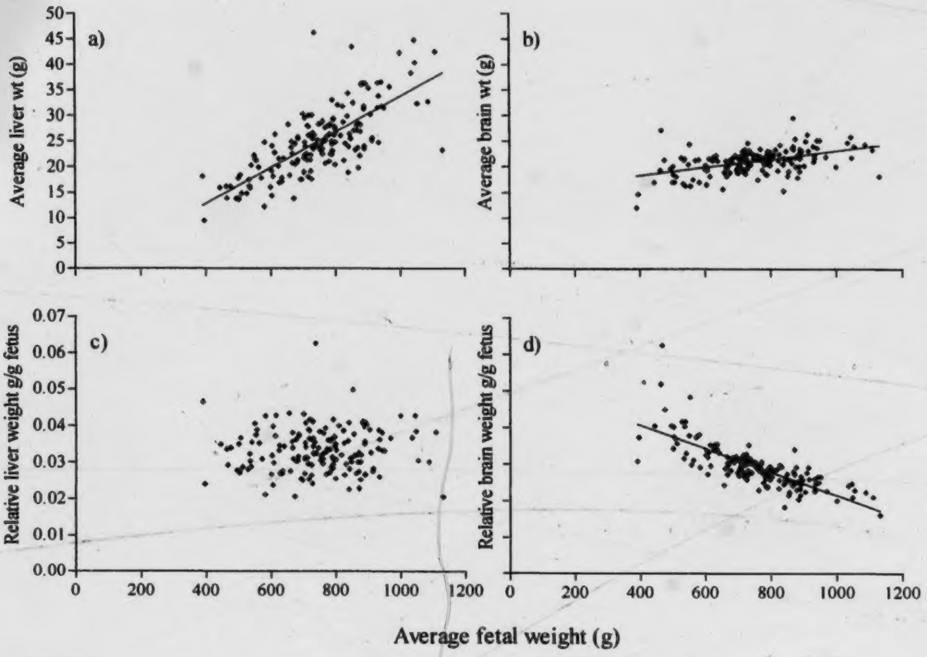
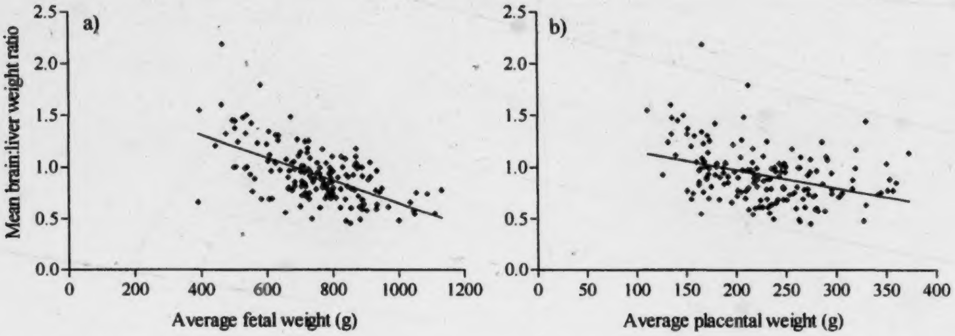


Figure 4.7 Relationship between mean brain:liver weight ratio and a) average fetal weight at day 85-90 of gestation (brain:liver wt ratio = 1.75 –0.0011(fetal wt), $R^2 = 0.35$, $P < 0.0001$) and b) average placental weight at day 85-90 of gestation (brain:liver wt ratio = 1.33 – 0.0018(placental wt), $R^2 = 0.14$, $P < 0.0001$). N = 166.



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CHAPTER FIVE

DEFINING THRESHOLDS FOR EFFECTS OF UTERINE CROWDING ON FETAL DEVELOPMENT*

5.1 INTRODUCTION

The largest proportion of prenatal loss in the pig has traditionally been viewed as occurring pre-implantation (as reviewed by Ashworth and Pickard, 1998). However, as discussed by Foxcroft (1997), the dynamics of prenatal loss in some dam-line genotypes appear to be changing, such that increasing numbers of embryos survive the implantation process, initially exceeding uterine capacity, but then die later in gestation. In commercial dam-line sows with a mean ovulation rate of 26.6, Vonnahme et al. (2002) identified the timing of the majority of post-implantation loss as occurring between days 30 and 50 of gestation. A similar sow population studied in Chapter 4 had a mean ovulation rate of 24.7, with approximately 15% of sows having greater than 30 ovulations, and Orzechowski (1998) reported mean ovulation rates of 26.9 in fifth parity sows of a different commercial dam-line. If these high ovulation rates were associated with even reasonable embryonic survival rates, the number of developing conceptuses in early gestation would greatly exceed uterine capacity.

The concept of uterine capacity has been widely studied in the pig using a variety of surgical models to examine the effects of crowding *in utero*. Dziuk (1968) concluded that when the number of embryos exceeds 14, intrauterine crowding was a limiting factor for litter size born. Fenton et al. (1970) determined that uterine capacity only becomes a limiting factor for fetal survival after day 25 of gestation, and Knight et al., (1977) further defined the critical period when uterine capacity exerts its effects to be between days 30 and 40 of gestation. Most of these earlier studies evaluated uterine capacity from the perspective of numbers born. However, Vallet (2000) reviewed evidence for crowding effects on placental development, consistent with data we have reported from both gilts (Almeida et al., 2002) and sows (Vonnahme et al., 2002). If an increase in placental efficiency does not adequately compensate for such limitations in placental size, fetal development of the surviving offspring could be affected in a manner analogous to intrauterine growth retardation (IUGR). Indeed, Town et al. (Chapter 6) reported negative effects on placental and fetal development, evidence of brain sparing (in terms of an increased brain:liver weight ratio as an indicator of IUGR, and a decrease in the number of secondary muscle fibres in day 90 fetuses from moderately crowded litters of third parity commercial dam-line sows.

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* A paper based on Chapter 5 by S.C. Town, C.T. Putman, W.T. Dixon and G.R. Foxcroft has been accepted subject to revision by Animal Reproduction Science.

The present study explored an alternative experimental approach to further examine whether variation in fetal numbers at early stages of gestation impacts fetal development. In this experimental paradigm, unilateral oviduct ligation of multiparous sows was used with the expectation that it would abrogate effects of uterine crowding resulting from high ovulation rates in higher parity sows, associated with high embryonic survival due to high health status.

5.2 MATERIALS AND METHODS

5.2.1 Animals

The experiment was conducted at the Swine Research and Technology Centre at the University of Alberta, in a controlled environment barn. Twenty-eight hybrid GP parity 4 to 6 sows (Genex Swine Group, Regina, Saskatchewan, Canada) were managed and fed as per standard protocols during gestation and lactation and were weaned at 21 days after farrowing. Average sow back fat measurement at weaning was 23 mm and body weight at post-weaning oestrus was 260 kg.

Ten sows were randomly allocated to be either an unmodified test control group (CTR test; n = 6) or a test ligation group (LIG test; n = 4), which underwent unilateral oviduct ligation surgery approximately 3 days after the end of their first post-weaning oestrus. The purpose of this surgery was to reduce the number of embryos *in utero* by preventing the oocytes ovulated from the ovary ipsilateral to the ligated oviduct from being fertilised and entering the uterus after breeding at the second post-weaning oestrus. The two test groups were killed at day 30 (mean day 29.2, range 27 to 30) of gestation to directly determine the number of conceptuses *in utero*. The remaining 18 sows were allocated to be either main trial control sows (CTR; n=11) which underwent embryo count surgeries at day 30 (mean day 27.3, range 26 to 33) of gestation to determine embryo number *in utero*, or ligated (LIG; n=7) sows which underwent unilateral oviduct ligation surgery only. As a welfare consideration, the decision was made to limit surgical intervention to a single surgical procedure per animal. Therefore, day 30 measures of embryo survival were not carried out on the ligated group. The main trial animals were killed at day 90 (mean day 88.7, range 85 to 94) of gestation to determine the effects of crowding on fetal development.

The original design of this experiment, was based on an expected availability of up to 50 sows for study, however, a shortage of available animals led to a more limited allocation of sows to treatment.

All experimental procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care and under authorization from the University of Alberta Animal Policy and Welfare Committee (approval #200046-D).

5.2.2 Embryo count surgery procedure

Sows were taken off feed for at least 12 hours before surgery but given free access to fresh water. The animals were restrained with a nose snare, then given an intravenous short acting general anaesthetic (5% solution of sodium thiopental, 'Pentothal', Merial Ltd, Iselin, New Jersey, USA; dosage: 6.6 ml/kg body weight) via an ear vein. Anaesthesia was maintained with a closed circuit system of inhalation general anaesthetic halothane ("fluothane") and oxygen via a face mask and nitrous oxide was also used in combination with halothane for more effective analgesia. Heart rate and respiration rate were monitored by a surgical technician. Anaesthetized sows were placed in dorsal recumbency on a U shaped table (cradle). Each limb was tied to the table. The surgical site was prepared and draped in accordance with established sterile surgical procedures in veterinary surgery with the additional use of an adhesive incise-drape ('steri-drape', 3M Healthcare, St Paul, Minnesota, USA) to seal the site of incision. The surgeons were gowned, gloved and masked.

An incision was made through the abdominal skin on the ventral midline, commencing 2 cm posterior to the umbilicus and extending 10 to 12 cm posteriorly. The subcutaneous fat was blunt dissected to the abdominal aponeurosis (junction of the conjoined abdominal musculature fascia), which was incised. The underlying layer of peritoneal fat and finally the peritoneum were then incised to gain access to the abdominal cavity. The gravid uterus was located and exteriorised in sections. The exposed tissues were kept covered and moist with sterile pads and pre-warmed sterile physiological saline throughout the operation.

Implantation sites were visualised and embryos were located by gentle palpation of the uterus. Each ovary was briefly exposed to record ovulation rate as the number of visible corpora lutea. Ovulation rate was reconfirmed at slaughter when the ovary was dissected, allowing a more accurate count of corpora lutea, which was subsequently used for analysis. A prophylactic antibiotic was administered (either 'Trivetrin', Coopers Agrofarm Inc., Ajax, Ontario, Canada or 'Borgal', Hoechst Roussel Vet Canada Inc., Regina, Saskatchewan, Canada, (both contain Trimethoprim 40mg and Sulfadoxine 200mg); dosage: 2ml into the abdominal cavity).

The peritoneum and musculature were closed in separate layers using absorbable sutures; the peritoneum using continuous cat gut sutures, the abdominal muscle layer (if off the midline), and the subcutaneous layer by interrupted cat gut sutures. The skin was closed with a continuous, absorbable 'vicryl' suture line. The sutured skin incision was sprayed with an antiseptic solution topical wound dressing, ('Boroform', Hoechst Roussel Vet, Warren, New Jersey, USA). The animal was given a prophylactic, broad spectrum, long acting antibiotic by single dose intramuscular injection ('Liquamycin LA 200'

(Oxytetracycline 200mg/ml), Pfizer Animal Health, Exton, Pennsylvania, USA; dosage: 1ml/10kg body weight or alternatively 'Biomycin' (Oxytetracycline 200mg/ml), Boehringer Ingelheim Animal Health Inc., St Joseph Missouri USA; at the same dosage). Any treatments administered were recorded, as per quality assurance guidelines.

Post-operatively, the animal was returned to a clean, heated, individual pen with free access to water to allow recovery from anaesthesia. Sows were regularly observed for complications and for evidence of a return to normal food and water intake. After surgery, sows were returned to the gestating sow feeding schedule as per SRU standard operating procedures. Sows were housed at the University of Alberta Metabolic Unit for one week post surgery and were subsequently returned to stall housing at the Swine Research Unit (SRU).

5.2.3 Oviduct ligation surgery procedure

Oviduct ligation surgery was carried out approximately three days after the end of post-weaning oestrus. The procedure in terms of anaesthesia, surgical approach and post-operative care was the same as that described for the embryo count surgery. Modifications included a smaller incision through the abdominal skin on the ventral midline, commencing 2 cm posterior to the umbilicus and extending only 7 to 8 cm posteriorly towards the inguinal region. Instead of exteriorising the entire reproductive tract, one ovary was located and exteriorised to locate the oviduct. The oviduct was looped approximately one centimetre on either side of the ampullary-isthmic junction in a U-shape, and tied off with two absorbable sutures to occlude the blood supply. After both ligatures were in place, the looped section of oviduct was removed to prevent reannealing of tissue. As with the embryo count surgery procedure, the exposed tissues were kept covered and moist with sterile physiological saline throughout the operation to prevent tissue adhesions. The ligated oviduct was replaced into its proper position inside the abdominal cavity and the body wall layers, peritoneum, muscle, subcutaneous layer and skin closed as previously described.

5.2.4 Heat checking, breeding and blood sampling

All sows were bred by artificial insemination at their second post-weaning oestrus. Heat detection was carried out every 12 h (0700 and 1900) using the back pressure test during periods of fence-line contact with a mature vasectomised boar starting on day 18 of the oestrous cycle. All sows were bred by artificial insemination using pooled semen from the same group of three fertile boars, using 3 billion sperm per dose, at 12 h and 36 h after onset of standing heat and then every 24 h until animals were no longer in standing heat (i.e. 12 h, 36 h, 60 h etc). At 72 h after the onset of oestrus, a blood sample was obtained from each animal by acute venepuncture of the ear vein during a brief period of nose-snare restraint for determination of circulating plasma progesterone concentrations. Signs of a return to oestrus were recorded between days 18 and 22 post-insemination and pregnancy was confirmed at day 25 of gestation using Real Time Ultrasound (RTU).

5.2.5 Progesterone radioimmunoassay

All samples were analysed in duplicate in a single assay using a 'Coat-a-Count' radioimmunoassay kit (DPC, Los Angeles, USA), previously validated for pig plasma (Mao and Foxcroft, 1998). The intra-assay CV was 6%, and the sensitivity of the single assay run was 0.1 ng/ml at 90% bound.

5.2.6 Slaughter and necropsy procedure

Sows were shipped to a local abattoir and reproductive tracts were recovered and dissected within one hour after slaughter. Ovulation rate and the number of viable embryos or fetuses *in utero*, were recorded for all sows. At day 30 of gestation, embryonic and placental weights and embryo crown-rump-lengths (CRL) were also measured. At day 90, fetal and placental weights were recorded and all fetuses were necropsied to determine various body organ weights including the brain, heart, lungs, liver, kidneys, spleen, pancreas and adrenals. After removal and weighing of the internal organs, the empty carcass was also weighed. The brain:liver weight ratio was then used as an estimate of proportional changes in organ development, indicative of the occurrence of IUGR. Results were averaged within litter. Relative piglet organ weights (actual organ weight divided by the actual body weight) were also calculated to further examine the pattern of organ growth in fetuses with different body weights. Again the average measurement from each litter was used for analysis.

To exclude extremes of fetal development, the two day 90 fetuses closest to the mean litter body weight were chosen for removal of the *semitendinosus* muscles, which were dissected, weighed, mounted on aluminium foil in a slightly stretched position and frozen in melting isopentane cooled in liquid nitrogen (-156°C). Samples were stored at -80°C until used for immunohistochemical and electrophoretic analyses.

5.2.7 Antibodies

The following monoclonal antibodies directed against various adult myosin heavy chain (MHC) isoforms were used: MF-20 (raised against all MHC isoforms; Developmental Studies Hybridoma Bank, University of Iowa, USA), NOQ7.5.4D (anti-MHCIβ; Sigma-Aldrich, Saint Louis, MO, USA), BA-D5 (anti-MHCIβ; Schiaffino et al., 1989), F88.12F8 (anti-MHCIa; Biocytex, Marseille, France), MY-32 (recognizes developmental and all fast MHC isoforms; Sigma-Aldrich, Saint Louis, MO, USA), SC-71 (anti-MHCIIa; Schiaffino et al., 1989), BF-F3 (anti-MHCIIb; Schiaffino et al., 1989) and BF-35 (recognizes all MHC isoforms except for MHCIId(x); Schiaffino et al., 1989). In order to detect developmental MHC isoforms, the following antibodies were used: NCL-d (anti-MHC embryonic) and NCL-n (anti-MHC neonatal) (Novocastra Laboratories, Newcastle, UK) and BF-45 (anti-MHC embryonic; Schiaffino et al., 1988). Secondary biotinylated horse anti-mouse IgG (rat-adsorbed and affinity-purified) and biotinylated goat anti-mouse IgM (used with BF-F3) were obtained from Vector Laboratories (Burlingame, CA, USA). Normal control mouse IgG was obtained from Santa Cruz Biochemical (CA, USA).

5.2.8 Immunohistochemical staining

10μm serial sections of fetal day 90 *semitendinosus* muscle were collected on poly-L-lysine coated slides (Electron Microscopy Sciences, Fort Washington, PA, USA) and stored at –80°C for later analysis. The avidin biotin peroxidase technique (Vectastain

Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) was used to visualise immunoreactivity. Frozen muscle sections were air-dried, washed once in phosphate-buffered saline with 0.1% (v/v) Tween 20 (PBS-Tween), twice in PBS, and incubated for 15 min in 3% (v/v) H_2O_2 in methanol. Sections were subsequently washed and incubated overnight at 4°C in the appropriate blocking solution (BS) containing 20% (v/v) Avidin D blocking solution (Vector Laboratories, Burlingame, CA, USA). (BS-1; 2.4% (w/v) protease-free BSA, 6% (v/v) Horse serum, and 0.1% (v/v) Tween 20 in PBS, pH 7.4). Sections were washed in PBS followed by incubation with the primary antibody for 1 h at room temperature in appropriate BS containing 20% (v/v) Biotin blocking solution (Vector Laboratories, Burlingame, CA, USA). Primary anti-mouse IgG monoclonal antibodies were diluted in BS-1 as follows: NOQ7.5.4D 1:4000; MY-32 (culture supernatant) 1:1000. Control sections were processed in parallel incubations in which the primary antibody was substituted with non-specific control mouse IgG (Santa Cruz, CA, USA). Sections were washed as before and incubated for 1 h at room temperature with the secondary antibody (biotinylated horse anti-mouse IgG diluted 1:500 in BS-1). Sections were then washed and incubated with Vectastain ABC reagent for 45 min at room temperature, washed again and reacted for 6 min with the substrate solution containing diaminobenzidine, H_2O_2, and $NiCl_2$ in 50 mmol/l Tris·HCl, pH 7.5 (Vector Laboratories DAB Substrate Kit for Peroxidase). The reaction was stopped by washing several times with distilled water. After dehydration in ethanol, sections were cleared with xylene and mounted with Entellan (Merck, Darmstadt, Germany).

Image acquisition was carried out using a motorised scanning stage Zeiss Axioplan IIM Universal Microscope (Carl Zeiss Jena GmbH, Jena, Germany) and a Photometrics CoolSNAP HQ Camera (Roper Scientific, Tucson, AZ, USA) in conjunction with the Metamorph Imaging System (Universal Imaging Corporation, Downingtown, PA, USA).

5.2.9 Myosin extraction

Frozen muscles were pulverised and extracted in six volumes of a buffer containing 0.3 mol/l KCl, 5 mmol/l $MgCl_2$, 5 mmol/l EGTA, 100 mmol/l $Na_4P_2O_7$, 1 mmol/l dithiothreitol and 5mg/ml Complete Mini Protease Inhibitor (Roche Diagnostics GmBH, Mannheim, Germany), pH 8.5. The solution was stirred for 30 min on ice and cleared by centrifugation for 5 min at 12,000 x g at 4°C. The supernatants were diluted 1:1 (v/v) with glycerol and stored at -20°C. Protein concentrations were determined using the Bradford procedure (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.10 Standard SDS-PAGE

The MHC complement of whole muscle extracts was analysed by SDS-PAGE using a slightly modified version (Putman et al., 2003) of the method described by Hämäläinen and Pette, (1996). Briefly, the separating gel contained 7% (w/v) polyacrylamide and 35% (v/v) glycerol, and the stacking gel was composed of 4% (w/v) polyacrylamide and 25% (v/v) glycerol. The upper buffer (25 mmol/l Tris-base, 192 mmol/l glycine, 10 mmol/l SDS) was supplemented with 0.2% (v/v) 2-mercaptoethanol. Before loading, extracts were incubated for 5 min at 100°C in a buffer containing 2.3% (w/v) SDS, 8% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 500 mmol/l Tris-base (pH 7.2), 0.1% (w/v) bromophenol blue and 44% (w/v) sucrose. Samples were cleared by centrifugation at 12,000 x g at 4°C, and 0.75 μg of total protein was loaded in each well. MHC isoforms were separated at 10°C for 24 h at 275 V (constant voltage) and visualised by silver staining. The relative MHC isoform contents were quantified densitometrically using the Syngene Chemigenius gel documentation system and GeneTools gel analysis software (Syngene, Cambridge, UK). The scheme used for identifying the various MHC isoforms was validated by Western blotting (Chapter 6) and by comparison with rodent muscles. Two piglets were analysed per litter and the results were averaged within sow to examine the effects of uterine crowding on muscle fibre development.

5.2.11 Statistical analysis

To determine the effects of treatment on ovulation rate, conception rate, number of viable embryos, embryonic survival rate, placental and embryo/fetal weights, placental efficiency, fetal organ weights, fetal brain:liver weight ratio, muscle weight and MHC isoform distribution, data were analysed as appropriate for a completely randomised design. To ensure the independence of observations, sow was used as the experimental unit for analysis, and fetal weights, placental weights, organ and muscle parameters were averaged within each reproductive tract (sow) before analysis.

Data were analysed within gestational time point (day 30 or day 90) by one-way ANOVA using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1990, SAS Inst. Inc., Cary, NC). Since sows were not slaughtered at exactly day 90, the model included treatment group (i.e. CTR or LIG) and gestation day at slaughter as a covariate if it was significant for the variable analysed. The gestation day x treatment group interaction effect was tested to determine if the slopes were equal. Again, if slopes were heterogeneous for the variable analysed and accounted for in the analysis, treatment groups were compared at the mean gestation day at slaughter (day 89). Significance was considered as $P < 0.05$. The results of the one-way ANOVA are presented as means ± SEM. If covariate (gestation day at slaughter) or interaction (gestation day x treatment group interaction) were significant, data are presented as LS means ± SEM.

Relevant associations within gestational age between number of viable embryos/fetuses, embryo/fetal weight, placental weight, placental efficiency, embryonic survival rate, plasma progesterone, fetal organ weights (day 90) and brain:liver weight ratio were examined using the INSIGHT procedure (SAS, 1990).

5.3 RESULTS

5.3.1 General results

Removals from final analysis were due to missing data as a result of damage during tract removal and tissue collection (missing ovaries, etc) and a non-pregnant sow in the test group. Total ovulation rate for this sow population (test and main trial animals; $N = 27$) was 18.07 ± 0.46 and was not different between CTR sows and LIG sows in either the test study or the main trial ($P = 0.28$ and $P = 0.93$, respectively; Table 5.1). Ovulation rates available for fertilization differed between the two treatment groups for both test and main trial animals ($P < 0.0001$; Table 5.1), and were positively associated with embryo numbers at day 30 ($R^2 = 0.67$; $P < 0.0001$; Figure 5.1a) and day 90 ($R^2 = 0.52$; $P < 0.001$; Figure 5.1b). The number of embryos at day 30 was strongly related to the number of fetuses at day 90 ($R^2 = 0.82$; $P < 0.0001$; Figure 5.2).

The number of viable embryos at day 30 was higher in the test CTR than in the test LIG sows ($P = 0.0002$), and in the main study the number of fetuses at day 90 was higher in the CTR compared to the LIG sows ($P = 0.02$; Table 5.1). Embryonic survival to day 30 was no different between test groups ($P = 0.57$); however, fetal survival to day 90 was higher in the LIG group ($P < 0.004$; Table 5.1). Average placental weight was no different between groups at either day 30 ($P = 0.83$) or day 90 of gestation ($P = 0.12$; Table 5.1). Embryonic weight and crown-rump-length measurements were not different between groups at day 30 ($P = 0.33$ and $P = 0.40$, respectively), and there was no difference between groups in fetal weights at day 90 ($P = 0.61$). Furthermore, placental efficiency, calculated as the embryonic weight: placental weight ratio, was no different between groups at either day 30 ($P = 0.1122$) or day 90 ($P = 0.1462$).

Placental weight was positively associated with embryonic weight at day 30 of gestation ($R^2 = 0.75$; $P = 0.0024$; test sows; Figure 5.3a) and with average fetal weight at day 90 of gestation ($R^2 = 0.38$; $P = 0.007$ main trial sows; Figure 5.3b). However, placental weight was not related to the number of viable embryos at day 30 ($P = 0.86$; Figure 5.4a) or with number of viable fetuses at day 90 ($P = 0.37$; Figure 5.4b), and there was no relationship between the number of fetuses and fetal weight at day 90 ($P = 0.58$; Figure 5.5). Placental efficiency was not associated with average fetal weight at day 90 of gestation ($P = 0.25$), or with the number of viable fetuses at day 90 ($P = 0.35$). Furthermore, placental efficiency showed no relationship with average placental weight at day 90 ($P = 0.57$).

5.3.2 Progesterone assay data

Sow plasma progesterone concentrations at 72 h after onset of standing heat ranged between 2.09 and 11.43 ng/ml and associations with embryonic survival to day 30 (using data from the test animals and from the Main trial CTR group) or fetal survival rate to day 90 (Main trial animals) are shown in Figure 5.6.

5.3.3 Day 90 necropsy data

Fetal organ weight data at day 90 of gestation are shown in Table 5.2. The pancreas was the only organ for which there was a difference between treatments, with an increase in weight in fetuses from CTR sows ($P = 0.015$). The brain:liver weight ratio was no different between fetuses from CTR and LIG sows ($P = 0.64$).

Fetal weight was positively related to absolute weight of the fetal liver ($R^2 = 0.32$; $P = 0.014$; Figure 5.7a), brain ($R^2 = 0.64$; $P < 0.0001$; Figure 5.7b), heart ($R^2 = 0.94$; $P < 0.0001$; Figure 5.8a), lungs ($R^2 = 0.86$; $P < 0.0001$; Figure 5.8b), spleen ($R^2 = 0.61$; $P < 0.0001$; Figure 5.8c), pancreas ($R^2 = 0.62$; $P < 0.0001$; Figure 5.9a), and kidneys ($R^2 = 0.48$; $P = 0.0013$; Figure 5.9b). There was no association with adrenal weight ($P = 0.11$; Figure 5.9c). When relative organ weights were calculated as the absolute organ weight:body weight ratio, mean relative heart weight showed a positive relationship with mean fetal weight ($R^2 = 0.32$, $P < 0.014$). No associations were evident ($P \geq 0.1$) with mean relative brain, liver, lung, spleen, pancreas, kidney and adrenal weights. The mean brain:liver weight ratio was neither related to mean fetal weight ($P = 0.78$; Figure 5.10a) nor mean placental weight ($P = 0.89$; Figure 5.10b), nor was it associated with the number of viable fetuses ($P = 0.45$; Figure 5.11). Average muscle weight was not significantly different between groups ($P = 0.68$; Table 5.2).

5.3.4 Immunohistochemical analysis

5.3.4.1 Myosin isoforms

Immunohistochemistry results are the same as those shown in Chapter 6 (Figure 6.11;f,h,l). For the day 90 fetal *semitendinosus* tissue, positive specific staining for MHCIβ (NOQ7.5.4D) was observed. MY-32 (all fast MHC) seemed to give a positive reaction with primary fibres in addition to positively staining secondary muscle fibres in day 90 tissue. MY-32 has previously been shown to react with swine MHCIα (Lefaucheur et al., 1995, 1997) and all fast MHC respectively (Fazarinc et al., 1995).

5.3.5 Standard SDS-PAGE

The MHC complement of whole muscle extracts of day 90 fetal tissue was analysed by SDS-PAGE and measured densitometrically. The electrophoretic separation of four different MHC isoforms was the same as shown in Chapter 6 (Figure 6.12). A comparison of the mobility of the four different MHC isoform bands with bands obtained from rat muscle, adult pig muscle and neonatal pig muscle was carried out, and the distribution of MHC isoforms is shown in Table 5.3. The fetal MHC isoform was most abundant, followed by embryonic, type IIa and type Iβ. There were no differences in relative isoform distribution between fetuses from LIG and CTR sows (Table 5.3).

5.4 DISCUSSION

In gilts and weaned first-parity sows in existing commercial dam-line genotypes, ovulation rate and early embryonic survival are probably still the key determinants of litter size. However, evidence for changing patterns of prenatal loss discussed by Foxcroft (1997) suggests that in some mature sow populations, high ovulation rates can be associated with relaxed selection among embryos in the pre-implantation period, resulting in a critical shift in the pattern of prenatal loss. In these sows, the number of conceptuses *in utero* around day 30 of gestation can greatly exceed uterine capacity. Based on existing literature, we hypothesised that this will have important consequences for subsequent development. Using other experimental paradigms we have obtained data to support this hypothesis (Chapter 3, Chapter 6). The objective of the present study was to evaluate the use of unilateral oviduct ligation in higher parity sows as an alternative experimental approach to this problem.

The first critical observation was that ovulation rate for the multiparous sow population studied was only 18.1 ± 0.46. Although this allows the possibility of substantially increased uterine crowding compared to the gilts studied by van der Lende et al. (1990) and in Chapter 3, this ovulation rate is still considerably lower than the ovulation rates of 26.9, 26.6 and 24.7 observed in the multiparous dam-line sows studied by Orzechowski (1998), Vonnahme et al. (2002) and in Chapter 4, respectively. Collectively, these data suggest important differences in key reproductive characteristics in existing commercial dam-line sows, presumably arising as an indirect response to selection programs mainly targeted at litter size. The lower ovulation rate in the present study may relate to the purebred status of the sow. However, as a mean ovulation rate of only 19.2 was observed in third parity F1 sows derived from the purebred sows used in the present experiment (Chapter 6), a lower ovulation rate may be characteristic of this particular commercial dam-line.

Evidence supporting the concept of progesterone-dependent mechanisms mediating embryonic survival has been reviewed previously (Foxcroft, 1997). Using the progesterone analysis method devised by Pharazyn (1992) and used more recently by van den Brand et al. (2000) a similar pattern of results is observed in the present study (Figure 5.6). Although there is no simple linear relationship between plasma progesterone concentrations at 72 h after onset of standing heat and embryonic survival at day 30, consistent with previous data (van den Brand et al., 2000), data is similarly distributed such that high concentrations of progesterone are not associated with lower levels of embryonic survival. Progesterone concentrations are in the same range (2.09 to 11.43 ng/ml) as those observed in a previous study (0.67 to 9.69 ng/ml;Chapter 6). Overall, progesterone concentrations in the present study are lower than those observed by Pharazyn (1992), although his studies used gilts subjected to nutritional manipulation rather than sows. Clearly the population of animals that have high embryonic survival and high progesterone levels are missing in the present study, perhaps due to the fact that these are higher parity purebred sows.

The increased number of viable embryos at day 30 and fetuses at day 90 in the CTR animals compared to the LIG group demonstrates that oviduct ligation effectively reduces embryo numbers *in utero*. Although embryonic survival to day 30 was not affected by oviduct ligation, fetal survival to day 90 was 21% higher in the LIG group, consistent with other observations (Chapter 6). The lower survival rate in the day 90 CTR animals may be due to a combination of factors. Surgical intervention at day 30 may decrease survival to day 90. However, estimated embryonic survival of only 74% at day 30 in the subset of CTR sows is consistent with our observations from Chapter 6 and suggests that reproductive tract capacity is already exerting a selection effect even with only 13.7 ± 0.6 viable embryos *in utero* at day 30 and 11.5 ± 0.7 fetuses by day 90. The embryonic survival rates of the ligated animals in the current population are clearly indicative of animals in good reproductive health. Thus, even with ovulation rates of around 20, higher embryonic and fetal survival rates in higher health status sows still results in measurable effects of uterine crowding during gestation. In contrast, the overcrowding effects seen in other populations of lower health status sows was largely driven by the relatively high ovulation rates present (Vonnahme et al., 2002 and Chapter 4).

Both the analysis of main treatment effects and the analysis of the association between the numbers of embryos or fetuses and placental weight, indicate the lack of any crowding effect on placental development. This is in contrast to previous data (Almeida et al., 2000; Vonnahme et al., 2002) and the results presented in Chapter 6 and appears to identify the threshold above which the number of offspring *in utero* creates negative effects on placental growth. Given the lack of treatment effects on placental development, the lack of significant treatment effect on embryonic weight at day 30, or fetal weight at day 90, was not unexpected.

Additionally, unlike previous data, there was no difference in placental efficiency between treatment groups in the present study at either day 30 (P = 0.112) or day 90 (P = 0.146). This suggests that a change in placental efficiency may be a compensatory mechanism that only exists when crowding detrimentally affects placental growth.

The increased absolute weight of the pancreas in fetuses from CTR animals was surprising, however, this may have been due to the fact that the pancreas was very difficult to dissect due to its diffuse and fragile structure. The pancreas weights of day 90 fetuses were not included in subsequent studies due to the difficulty and inaccuracy of dissection and measurement of this organ (Chapter 6). Observed positive correlations between absolute fetal organ weights and body weight were expected. Although in other studies, increased numbers of embryos/fetuses *in utero* limited placental growth, and were strongly and negatively correlated with both fetal weight and placental weight (Chapter 6), none of these correlations were observed in the present study. Furthermore, the brain:liver weight ratio was no higher in fetuses from CTR litters and there was no correlation between brain:liver weight ratio and the number of viable fetuses, whereas a strong positive correlation has been observed in Chapter 3. Clearly, brain sparing, as measured by a high brain:liver weight ratio and indicative of IUGR (Bauer et al., 1998), was not occurring in the present population of animals.

The relative MHC isoform distribution results for day 90 fetal samples, with the fetal MHC isoform being most abundant, is consistent with the relative distribution of MHC isoforms reported in Chapter 6. The lack of an effect of uterine crowding on isoform distribution pattern is also in agreement with Chapter 6, in which the isoform distribution pattern was also unchanged, even though a higher level of uterine crowding resulted in a significant decrease in the number of fetal secondary muscle fibres.

In the present study, therefore, as well as being below the threshold for detrimental effects on placental development, the moderate uterine crowding observed in CTR sows in the early gestation did not exert effects on development of the surviving fetuses in a manner analogous to IUGR. On the basis that no brain sparing effect was observed, and that there was no difference in average muscle weight between the two treatment groups, a detailed analysis of muscle fibre numbers was not performed. However, it is expected that no differences in muscle fibre numbers would have been observed, in contrast to the results in Chapter 6 where day 90 fetuses from larger litters exhibited a decrease in secondary fibre numbers. The positive specific staining observed in day 90 fetal semitendinosus tissue for MHCIβ (using antibody NOQ7.5.4D), was in agreement with results from other studies (Chapter 6). As with these previous results, antibody MY-32 (all fast MHC) also seemed to give a positive reaction with primary fibres in addition to positively staining secondary muscle fibres. MY-32 has previously been shown to react with swine MHCIα (Lefaucheur et al., 1995, 1997) and all fast MHC respectively (Fazarinc et al., 1995). However, since MHCIα was shown not to be present in the semitendinosus muscle until after birth in the pig (Lefaucheur et al., 2001; Chapter 6). It is likely that MY32 cross reacts with MHCIβ rather than the possibility that primary fibres contained both MHCIα and Iβ.

Despite some limitation on the number of sows available, these data do provide further evidence for the biological basis of variability in postnatal growth performance. Collectively, recent studies indicate that the potential for uterine crowding to impact fetal development depends on an interaction between parity, genotype and the health status of the sow population (Chapters 3, 4 and 6). Members of the present population of sows have a fairly modest ovulation rate (approximately 18) coupled with a high embryonic survival rate (approximately 84%). Furthermore, based on parity effects on uterine and placental function reported in Chapter 4, the sows in the present study would be expected to be at peak reproductive performance in terms of functional uterine capacity and placental efficiency. The result is a reasonable balance between the number of surviving conceptuses with normal placental development and uterine capacity, such that the threshold for effects of uterine crowding on fetal development is not exceeded.

By comparison, although a relatively low ovulation rate (15.6 ± 0.6) and a lower embryonic survival rate (65.2 ± 4.9%) resulted in a mean of only 10 ± 0.8 embryos *in utero* at day 30, and a litter size born of 8.5 ± 0.7 pigs, in the gilt population studied in Chapter 3, uterine capacity was clearly more limiting in these animals. A moderate effect on birth weight, and brain sparing effects in surviving neonates were observed that would likely have contributed to differences in postnatal growth performance. Similarly, in third parity F1 sows studied in Chapter 6, even relatively modest uterine crowding produced negative effects on placental and fetal development, including a reduction in muscle size and in the number of secondary muscle fibres in day 90 fetuses. In genotypes with relatively high ovulation rates, even greater impacts on fetal development can be expected in that population of sows that have minimal embryonic loss to day 30 of gestation. This effect may be further exaggerated in high parity sows in which placental development appears to become compromised in later gestation (Chapter 4), effectively increasing the imbalance between the number of conceptuses and functional uterine capacity.

With a better understanding of critical reproductive characteristics of existing commercial dam-line sows, including interactions of health status and parity, weaned pigs could be segregated on the basis of anticipated developmental potential, rather than co-mingling offspring from gilts with litters from mid or higher parity sows. On this basis, maximal growth potential would be expected from the offspring derived from the sows used in the present study.

Table 5.1 Reproductive characteristics (means ± SEM) of Test sows (N = 10) and Main Trial sows (N = 18) in control (CTR) and unilaterally oviduct-ligated (LIG) sows.

	TREATMENT GROUP	
	Test Sows	
Parameter	**CTRtest (n=6) "Relatively Crowded"**	**LIGtest (n=4) "Non-Crowded"**
Overall ovulation rate	18.2 ± 0.7	16.5 ± 1.5
Ovulation rate available for fertilization	18.2 ± 0.7^{a}	7.0 ± 1.1^{b}
Number of viable embryos at d30	15.0 ± 0.9^{a} (n = 6)	5.3 ± 0.3^{b} (n = 3)
Embryonic survival to d30 (%)	83.5 ± 6.8 (n = 6)	90.5 ± 9.5 (n = 3)
Average placental weight (g) at d30	26.3 ± 0.6 (n = 6)	25.8 ± 1.1 (n = 3)
Average embryo weight (g) at d30	1.50 ± 0.2 (n = 6)	1.14 ± 0.2 (n = 3)
Embryonic crown-rump-length at d30 (mm)	24.1 ± 1.4 (n = 6)	22.2 ± 0.9 (n = 3)
Placental efficiency at d30	0.062 ± 0.005 (n = 6)	0.048 ± 0.004 (n = 3)
	Main Trial Sows	
	CTR (n=11) "Relatively Crowded"	**LIG (n=7) "Non-Crowded"**
Overall ovulation rate	18.5 ± 0.7 (n = 11)	18.3 ± 1.1 (n = 6)
Ovulation rate available for fertilization	18.5 ± 0.7^{a} (n = 11)	10.2 ± 1.0^{b} (n = 6)
Number of viable embryos (d30)	$13.7 \pm 0.6^{a*}$ (n = 11)	-
Fetal survival to d30 (%)	74.1 ± 3.0^{a} (n = 11)	-
Number of viable fetuses (d90)	11.5 ± 0.7^{a}	8.7 ± 1.0^{b}
Fetal survival to d90 (%)	62.4 ± 3.3^{a}	83.6 ± 5.8^{b}
Average placental weight (g) at d90	255.3 ± 13.5	296.7 ± 23.4
Average fetal weight (g) at d90	$599.3 \pm 14.3^{*}$	$592.7 \pm 18.3^{*}$
Placental efficiency at d90	$2.42 \pm 0.07^{*}$	$2.24 \pm 0.09^{*}$

Means ± SEM within a row with different superscripts differ ($P < 0.05$)
LS means ± SEM are marked with an asterisk (*)

Table 5.2 Average empty carcass and body organ weights and brain:organ weight ratios (means ± SEM) in fetuses from control (CTR) and unilaterally oviduct-ligated (LIG) sows (N=18) at day 90 of gestation.

	Treatment group	
Parameter	**CTR (n=11)** **"Relatively Crowded"**	**LIG (n=7)** **"Non-Crowded"**
Spleen (g) (LS Mean)	0.83 ± 0.03*	0.77 ± 0.03*
Liver (g)	18.22 ± 1.17	17.85 ± 1.02
Heart (g) (LS Mean)	4.02 ± 0.11*	3.92 ± 0.14*
Lungs (g) (LS Mean)	16.82 ± 0.44*	16.15 ± 0.56*
Kidneys (g)	6.06 ± 0.31	6.23 ± 0.23
Pancreas (g) (LS Mean)	0.93 ± 0.02^{a}*	0.84 ± 0.03^{b}*
Adrenal (g)	0.11 ± 0.01	0.10 ± 0.003
Brain (g) (LS Mean)	19.18 ± 0.31*	18.84 ± 0.39*
Semitendinosus muscle (g) (LS Mean)	2.08 ± 0.06*	2.04 ± 0.08*
Empty carcass (g) (LS Mean)	474.4 ± 11.7*	473.44 ± 15.0*
Brain:Liver wt ratio	1.09 ± 0.07	1.14 ± 0.08
Brain:Muscle wt ratio	9.20 ± 0.27	9.29 ± 0.32

Means ± SEM within a row with different superscripts differ ($P < 0.05$)
LS means ± SEM are marked with an asterisk *

Table 5.3 Myosin Heavy Chain Isoform distribution (mean % ± SEM) in day 90 fetal *semitendinosus* muscle from control (CTR) and unilaterally oviduct-ligated (LIG) sows (N=18).

	Treatment group	
Parameter	**CTR (n=10)** **"Relatively Crowded"**	**LIG (n=7)** **"Non-Crowded"**
Embryonic MHC (%)	34.60 ± 5.26	24.54 ± 2.36
Fetal MHC (%)	45.12 ± 5.81	56.65 ± 2.29
Type IIa MHC (%)	13.47 ± 0.79	12.62 ± 1.24
Type Iβ MHC (%)	6.80 ± 1.54	6.18 ± 1.56

Figure 5.1 Relationship between ovulation rate available for fertilization and a) number of viable embryos at day 30 of gestation (number of embryos at day 30 = 2.65 + 0.612(available ovulation rate), $R^2 = 0.67$; $P < 0.0001$; n = 21) in LIG test trial (◇), test CTR (◆) and main trial CTR (○) animals and b) number of viable fetuses at day 90 of gestation (number of fetuses at day 90 = 4.12 + 0.405(available ovulation rate), $R^2 = 0.52$; P = 0.0011; main trial sows; n = 17) in LIG animals (◇) and CTR animals (◆).

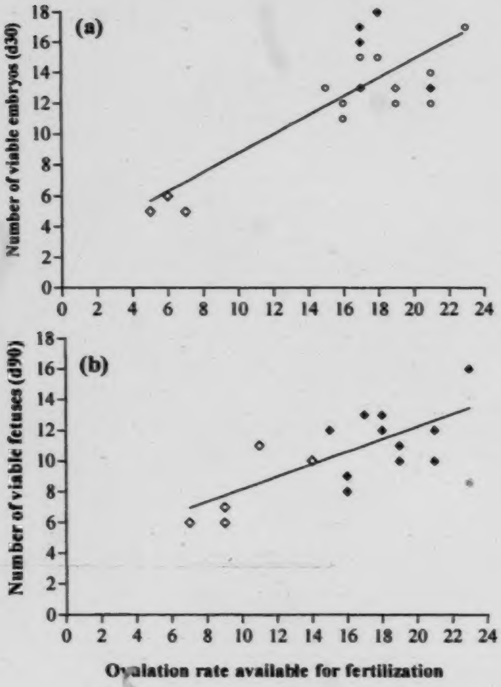


Figure 5.2 Relationship between the number of viable embryos at day 30 of gestation and the number of viable fetuses at day 90 of gestation (number of fetuses at day 90 = -1.08 + 0.931(number of embryos at day 30), $R^2 = 0.82$; $P < 0.0001$; n = 17 main trial sows) in LIG animals (◇) and CTR animals (◆). A 100% conception rate is assumed in ligated animals in which embryo count surgeries were not carried out.

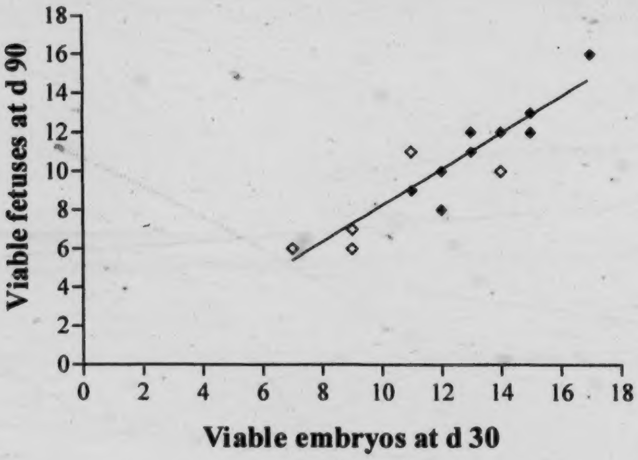


Figure 5.3 Relationship between average placental weight and a) average embryonic weight at day 30 of gestation (placental wt = 3.80 + 16.22(embryo wt at day 30), $R^2 = 0.75$; $P = 0.0024$; test sows; n = 9) and b) average fetal weight at day 90 of gestation (placental wt = 22.51 + 0.42(fetal wt at day 90), $R^2 = 0.38$; $P = 0.007$ main trial sows; n = 18) and in LIG animals (◇) and CTR animals (◆).

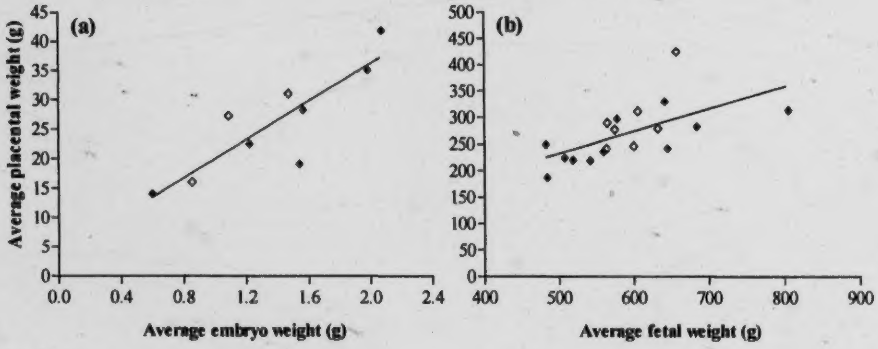


Figure 5.4 Lack of a relationship between placental weight and a) number of viable embryos at day 30 (P = 0.86; test sows; n = 9) and b)number of viable fetuses at day 90 (P = 0.37; main trial sows; n = 18) in LIG (◇) and CTR animals (◆).

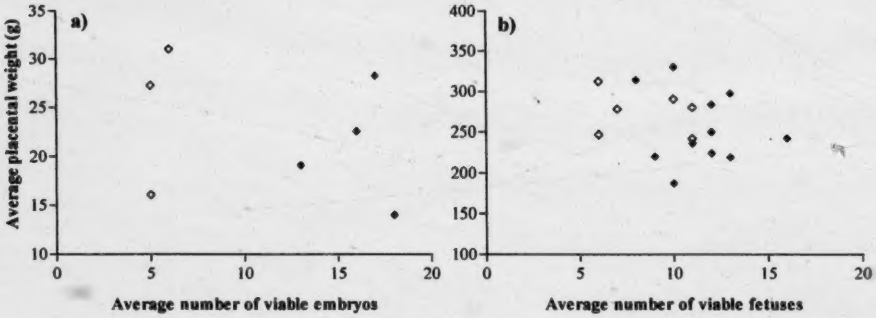


Figure 5.5 Lack of a relationship between the number of viable fetuses and fetal weight at day 90 (P = 0.58) in LIG (◇) and CTR animals (◆).

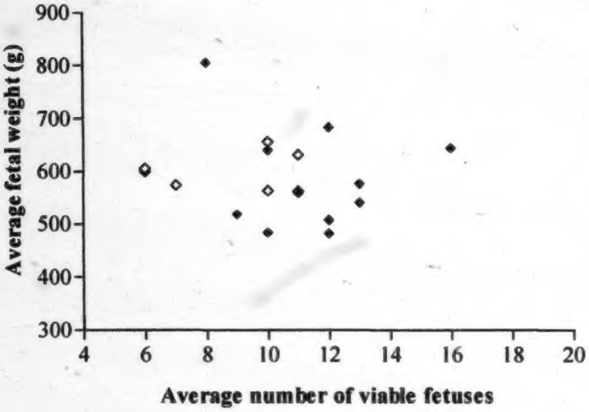


Figure 5.6 Lack of a relationship between plasma progesterone at 72 hr after the onset of standing heat (ng/ml) and (a) embryonic survival rate (%) at day 30 of gestation (P = 0.614; n = 21) in LIG test trial (◇), test CTR (◆) and main trial CTR (○) animals and (b) fetal survival rate (%) at day 90 of gestation (P = 0.994; n = 17; main trial sows) in LIG animals (◇) and CTR animals (◆). The horizontal lines represent the average embryonic survival on day 30 (79%) and day 90 (70%) and the vertical lines are arbitrarily drawn.

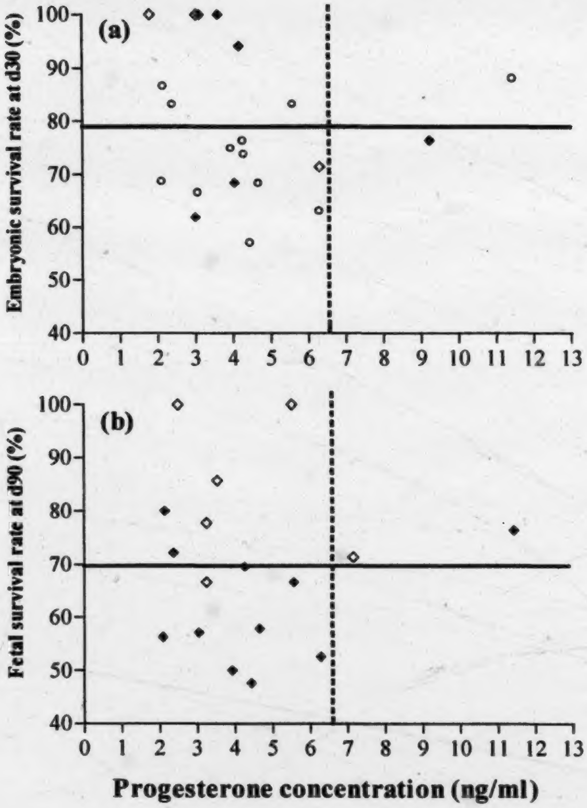


Figure 5.7 Relationship between (a) mean absolute liver weight (liver wt = 3.78 + 0.024(fetal wt), $R^2 = 0.32$; $P = 0.0141$) and (b) mean absolute brain weight (brain wt = 4.22 + 0.025(fetal wt), $R^2 = 0.64$; $P < 0.0001$) and average fetal weight at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

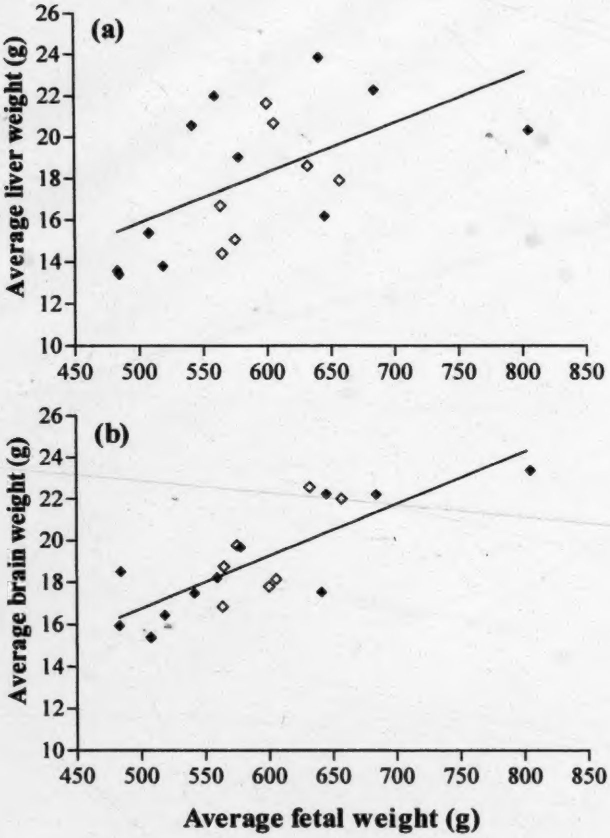


Figure 5.8 Relationship between (a) mean absolute heart weight (heart wt = - 0.87 + 0.008(fetal wt), $R^2 = 0.94$; $P < 0.0001$) (b) mean absolute lung weight (lung wt = 0.94 + 0.026(fetal wt), $R^2 = 0.86$; $P < 0.0001$) and (c) mean absolute spleen weight (spleen wt = 0.15 + 0.001(fetal wt), $R^2 = 0.61$; $P < 0.0001$), and average fetal weight at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

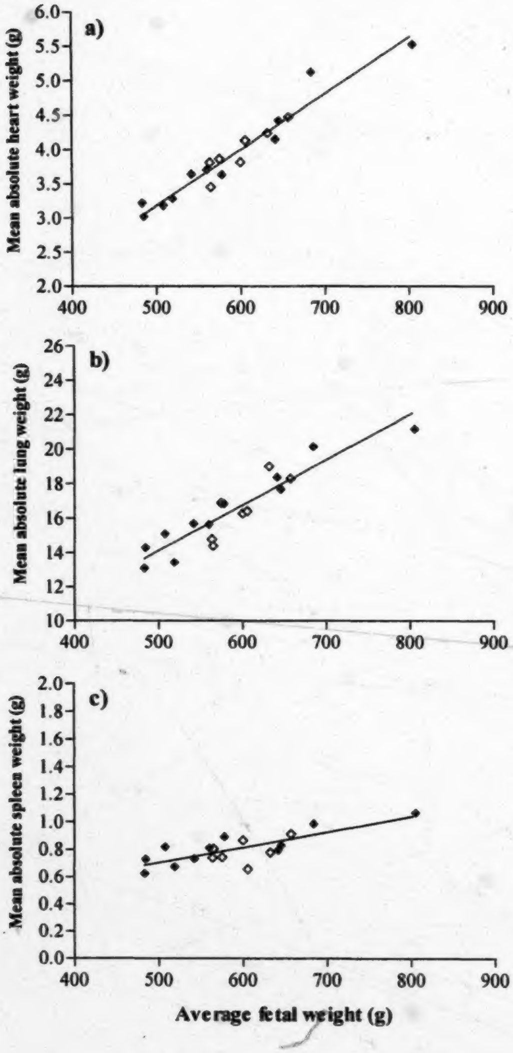


Figure 5.9 Relationship between (a) mean absolute pancreas weight (pancreas wt = 0.25 + 0.001(fetal wt), $R^2 = 0.62$; $P < 0.0001$) (b) mean absolute kidney weight (kidney wt = 1.58 + 0.008(fetal wt), $R^2 = 0.48$; $P = 0.0013$) and (c) mean absolute adrenal weight ($P = 0.11$), and average fetal weight at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

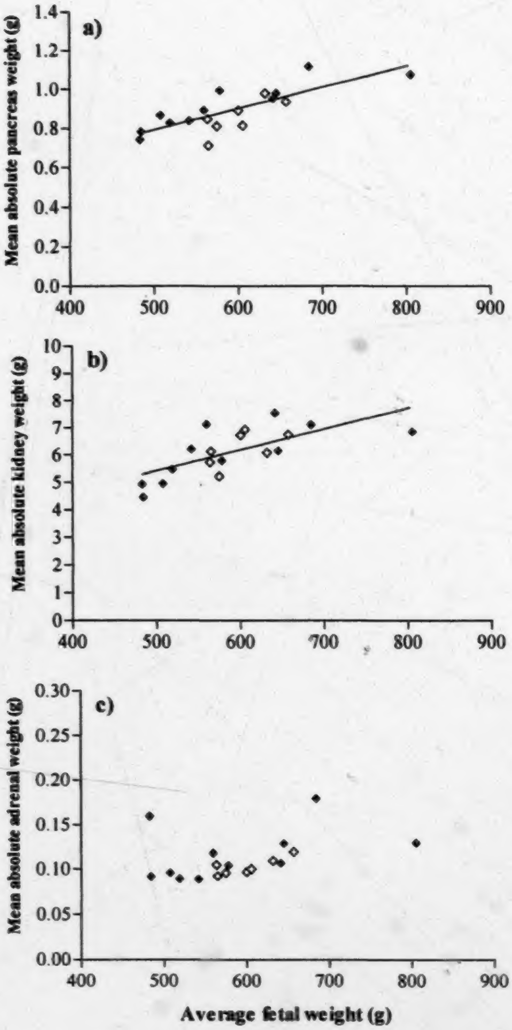


Figure 5.10 Lack of a relationship between the mean brain:liver weight ratio and a) mean fetal weight (P = 0.78) and b) mean placental weight (P = 0.89) at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

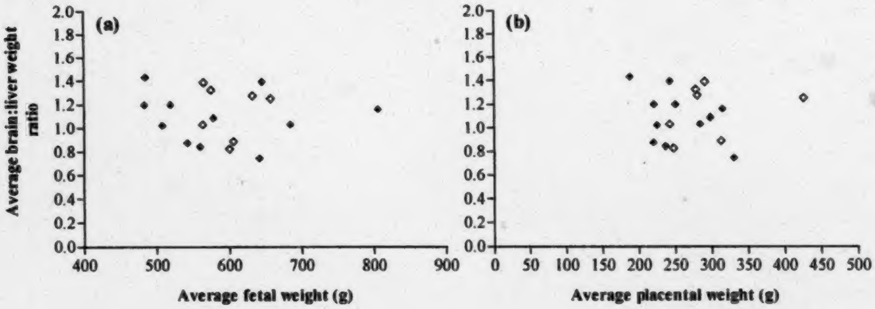
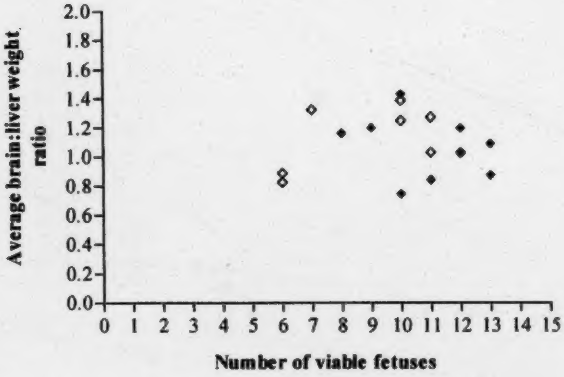


Figure 5.11 Lack of a relationship between the mean brain:liver weight ratio and, the number of viable fetuses (P = 0.45) at day 90 of gestation in LIG animals (◇) and CTR animals (◆).



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CHAPTER SIX

NUMBER OF CONCEPTUSES *IN UTERO* AFFECTS PORCINE FETAL MUSCLE DEVELOPMENT[*]

6.1 INTRODUCTION

The regulation of prenatal survival in litter bearing mammals like the rat, rabbit and pig has been extensively studied. In domestic species like the pig, the number of offspring born is an important economic trait, and the components of litter size (ovulation rate, embryonic survival and uterine capacity) responsive to genetic selection are well established (Johnson et al., 1985). However, as selection for ovulation rate has been associated with selection against early embryonic survival, and because birth weight decreased as litter size increased, it was concluded that selection for uterine capacity might be the most productive selection parameter (Johnson et al., 1999). A recent study of associations among within-litter variation in birth weight, and pre-weaning survival and weight gain, also led to the conclusion that selection for increased litter size that results in more low-birth-weight piglets may not be beneficial, unless measures are taken to improve the survival of the low-birth-weight offspring (Milligan et al., 2002). Thus, both the developmental competence of the pigs born, and the size of the litter, need critical consideration.

The concept of uterine capacity as the ultimate limitation on litter size was established using different animal models to study effects of uterine crowding in the pig (uterine ligation, oviduct resection, unilateral hysterectomy and ovariectomy (UHO), superovulation, and embryo transfer), leading to the conclusion that when the number of embryos exceeded 14, intrauterine crowding was a limiting factor for litter size born (Dziuk, 1968). Bazer et al. (1969 a and b) also concluded that increased embryonic loss, associated with a greater number of embryos *in utero*, was due to maternal limitations and not to inherent limitations of the embryo. They suggested that two physiological mechanisms might be involved. Initially, embryo selection may occur as the result of competition among embryos for some biochemical factor in the uterus necessary for their continued development. In later gestation, intrauterine competition for the establishment of adequate surface area for nutrient exchange between fetal and maternal circulations may act to limit litter size. Interestingly, in the context of variation in development *in utero*, the concept has been advanced that mechanisms promoting competition among embryos in the pre-implantation period will act to reduce within-litter variation in development by selectively removing the least developed embryos (van der Lende et al., 1990). Nevertheless, the more recent results of Père et al. (1997) confirm that, even in sows with "normal" ovulation rates, uterine capacity can affect both litter size and the average birth weight of the litter.

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[*] A paper based on Chapter 6 by S.C. Town, C.T. Putman N.J. Turchinsky, W.T. Dixon and G.R. Foxcroft has been accepted subject to revision by Reproduction.

Fenton et al. (1970) determined that uterine capacity only becomes a limiting factor for fetal survival after day 25 of gestation. Knight et al. (1977) further defined day 30 to 40 of gestation as the critical period when uterine capacity exerts its effects, and subsequent studies in both intact and UHO females support this conclusion (see Vallet, 2000). Furthermore, based on a number of studies, Vallet et al. (2003) suggested that fetal growth rate is less sensitive to intrauterine crowding than placental growth rate and that, as in the prolific Meishan female (Ford and Youngs, 1993), an increase in placental efficiency may initially protect the developing fetus from a limitation in placental size. However, conclusions based only on a consideration of fetal weight may overlook critical effects on fetal development that are established early in gestation.

The study of within-litter variation in prenatal development suggested that the extremes of intrauterine growth retardation (IUGR) or "runting" described previously in the pig (Adams, 1971; Widdowson, 1971; Cooper et al., 1978; Hegarty and Allen, 1978; Flecknell et al., 1981), were identified within a discrete sub-population of fetuses (Royston et al., 1982; Wooton et al., 1983). In subsequent studies of the association between within-litter differences in prenatal development and postnatal survival and growth, van der Lende and de Jager (1991) concluded that the lower pre-weaning growth of the disadvantaged pigs born could not be entirely explained on the basis of their lower birth weight. This suggested that IUGR had a more complex effect on the developmental potential of such pigs. Interestingly, data from the same laboratory indicated that the extent of IUGR within a litter was associated with specific patterns of embryonic survival (van der Lende et al., 1990) and the largest litters *in utero* generally included one or more IUGR fetuses. Furthermore, the data from this study supported the conclusion that within-litter variation in development was already established at the early fetal stage (day 27 to 35) of gestation.

Pre-implantation embryonic losses are still considered to be the largest proportion of prenatal loss in the pig, although the number of fetuses post-implantation will ultimately reflect uterine capacity (as reviewed by Ashworth and Pickard, 1998). In commercial practice, this generalisation likely reflects the situation in gilts, and in weaned, first parity, sows that tend to be in a catabolic state. However, the dynamics of prenatal loss in some dam-line populations may be changing (Foxcroft, 1997). It appears that in some commercial dam-lines, several generations of selection for prolificacy has produced a discrepancy between the magnitude of the increase in ovulation rate and the number of conceptuses surviving post-implantation, and uterine capacity. As a consequence, the number of embryos surviving the pre-implantation stages of pregnancy initially greatly exceeds uterine capacity, and an increasing proportion of prenatal loss is now occurring in the post-implantation period.

Even in individual gilts with 20 or more ovulations, embryonic survival rate can be 100% at day 28 of gestation (Almeida et al., 2000), whereas average first litter size is still only 10 to 12 piglets. In higher parity females, the situation may be even more extreme and mean ovulation rates of 26.9 ± 1.4 (Orzechowski, 1998), 26.6 ± 0.4 (Vonnahme et al., 2002) and 24.7 ± 0.4 (Chapter 4) have been reported in some commercial dam-lines, with approximately 15% of these sows having greater than 30 ovulations. Despite relatively poor embryonic survival of approximately 60% to day 30 in these studies, the number of conceptuses *in utero* at day 30 (approximately 15) still probably exceeded uterine capacity. Consistent with literature reviewed earlier, Vonnahme et al. (2002) reported a significant reduction in conceptus number by day 45 to 50 of gestation. Furthermore, uterine crowding in the immediate post-implantation period was associated with a decrease in placental volume (Almeida et al., 2000) and placental weight (Vonnahme et al., 2002). Although the size and weight of the embryo was not affected by crowding up to day 44 of gestation, potential impacts on fetal development need careful study. If placental compensatory mechanisms are not adequate, crowding of the uterus in the early post-implantation period of gestation may affect fetal development of surviving conceptuses, in a manner analogous to IUGR. This raises important questions for both fetal and postnatal development and commercial growth performance, particularly with respect to the development of fetal muscle fibres, which start to differentiate around day 35 of gestation in the pig, i.e. within this critical "window".

In contrast to situations in which the occurrence of IUGR is limited to a discrete subpopulation of "runt" fetuses (Royston et al., 1982; Wooton et al., 1983), a changing pattern of embryonic loss that results in uterine crowding in early gestation appears to produce a more uniform effect on placental development that will thus affect the development of all surviving fetuses.

Based on data from experiments using the guinea pig, Dwyer et al. (1992) suggested that a reduction in placental size might be the mechanism mediating effects of maternal under-nutrition on fetal growth. Furthermore, a series of studies in the pig have demonstrated that maternal nutrition during gestation has an effect on piglet birth weight, and that low birth weight is primarily associated with a reduced number of secondary muscle fibres (Handel and Stickland, 1987; Dwyer et al., 1994). Consistent with earlier data of Hegarty and Allen (1978) indicating that runts in the litter have reduced muscle growth potential, Dwyer et al. (1993) also established a positive correlation between the total number of muscle fibres and postnatal growth potential, and that littermates with a high numbers of fibres grew faster and more efficiently than littermates with a lower number of fibres. Dwyer et al. (1994) further demonstrated that the detrimental effect of maternal undernutrition occurred between 25 and 50 days of gestation, the period immediately preceding secondary muscle fibre hyperplasia. This led to the central hypothesis tested in the present study, that, "by detrimentally affecting placental size in early gestation, uterine crowding will also affect fetal organ development and the number and type of muscle fibres, analogous to the situation of IUGR in nutritionally-challenged sows".

As preliminary data from an initial experiment indicated that even when the number of conceptuses *in utero* does not significantly affect birth weight, "crowding" nevertheless results in measurable IUGR in the fetus (Town et al., 2002), the present study evaluated an alternative experimental approach to further test our central hypothesis.

6.2 MATERIALS AND METHODS

6.2.1 Animals

The experiment was conducted at the Swine Research and Technology Centre at the University of Alberta, in a controlled environment barn during July to October 2001. Sixty Hybrid F1 third parity sows (Genex Swine Group, Regina, Saskatchewan, Canada) were managed and fed as per standard protocols during gestation and lactation and were weaned at 23 days after farrowing. Average sow body weight and back fat measurements at post-weaning oestrus were 210 ± 2.01 kg and 17.4 ± 0.33 mm, respectively. Backfat thickness was measured at the last rib, 6cm off the midline (P_2) (Renco Lean-Meter, Renco Corp., Minneapolis, USA). Sows were randomly allocated to one of two groups. The experimental group (n=30) underwent unilateral oviduct ligation surgery (LIG) approximately 3 days after the end of their first post-weaning oestrus. The purpose of this surgery was to reduce the number of embryos *in utero* by preventing the oocytes ovulated from the ovary ipsilateral to the ligated oviduct from being fertilised and entering the uterus. The remaining animals (n=30) did not undergo surgery and formed the control group (CTR). Half the animals from each group were killed at day 30 of gestation to determine the number of conceptuses *in utero*. The remaining animals were killed at day 90 of gestation to determine the effects of crowding on fetal development.

All experimental procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care and under authorization from the University of Alberta Animal Policy and Welfare Committee (approval #200134D).

6.2.2 Oviduct ligation surgery

Oviduct ligation surgery was carried out approximately three days after the end of post-weaning oestrus. The procedure in terms of anaesthesia, surgical approach and post-operative care was the same as that described for the final surgery ultrasound protocol (Chapter 3.2.3.2). Modifications included a smaller incision through the abdominal skin on the ventral midline, commencing 2 cm posterior to the umbilicus and extending only 7 - 8 cm posteriorly. Instead of exteriorising the entire reproductive tract, one ovary was located and exteriorised to locate the oviduct. The oviduct was looped approximately one centimetre on either side of the ampullary-isthmic junction in a U-shape, and tied off with two absorbable sutures to occlude the blood supply. After both ligatures were in place, the looped section of oviduct was removed to prevent reannealing of tissue. As with the embryo count surgery procedure, the exposed tissues were kept covered and moist with sterile physiological saline throughout the operation to prevent tissue adhesions. The ligated oviduct was replaced into its proper position inside the abdominal cavity and the body wall layers, peritoneum, muscle, subcutaneous layer and skin closed as previously described.

6.2.3 Heat checking, breeding and blood sampling

All sows were bred by artificial insemination at their second post-weaning oestrus. Heat detection was carried out every 12 h (0700 and 1900) using the back pressure test during periods of fence-line contact with a mature vasectomised boar starting on day 18 of the oestrous cycle. All sows were bred by artificial insemination using pooled semen from the same group of three fertile boars, using 3 billion sperm per dose, at 12 h and 36 h after onset of standing heat and then every 24 h until animals were no longer in standing heat (i.e. 12 h, 36 h, 60 h etc). At 72 h after the onset of oestrus, a blood sample was obtained from each animal by acute venepuncture of the ear vein during a brief period of nose-snare restraint for determination of circulating plasma progesterone concentrations. Signs of a return to oestrus were recorded between days 18 and 22 post-insemination and pregnancy was confirmed at day 25 of gestation using Real Time Ultrasound (RTU).

6.2.4 Progesterone radioimmunoassay

All samples were analysed in duplicate in a single assay using a 'Coat-a-Count' radioimmunoassay kit (DPC, Los Angeles, USA), previously validated for pig plasma (Mao and Foxcroft, 1998). The intra-assay CV was 6%, and the sensitivity of the single assay run was 0.1 ng/ml at 90% bound.

6.2.5 Slaughter and necropsy procedure

Sows were shipped to a local abattoir and reproductive tracts were recovered and dissected within one hour after slaughter. Ovulation rate and the number of viable embryos or fetuses *in utero*, were recorded for all sows. At day 30 of gestation, embryonic and placental weights were also measured. At day 90, fetal and placental weights were recorded and all fetuses were necropsied to determine various body organ weights including the brain, heart, lungs, liver, kidneys, and spleen. After removal and weighing of the internal organs, the empty carcass weight was also recorded. The brain:liver weight ratio was then used as an estimate of proportional changes in organ development, indicative of the occurrence of IUGR. Results were averaged within litter. Relative piglet organ weights (actual organ weight divided by the actual body weight) were also calculated to further examine the pattern of organ growth in fetuses with different body weights. Again the average measurement from each litter was used for analysis.

Two day 90 fetuses closest to the mean litter body weight were chosen for removal of the *semitendinosus* muscles, which were dissected, weighed, mounted on aluminium foil in a straightened position and frozen in melting isopentane cooled in liquid nitrogen (-156°C). Samples were stored at -80°C until used for immunohistochemical and electrophoretic analyses.

6.2.6 Antibodies

The following monoclonal antibodies directed against various adult myosin heavy chain (MHC) isoforms were used: MF-20 (raised against all MHC isoforms; Developmental Studies Hybridoma Bank, University of Iowa, USA), NOQ7.5.4D (anti-MHCIβ; Sigma-Aldrich, Saint Louis, MO, USA), BA-D5 (anti-MHCIβ; Schiaffino et al., 1989), F88.12F8 (anti-MHCIa; Biocytex, Marseille, France), MY-32 (recognizes developmental and all fast MHC isoforms; Sigma-Aldrich, Saint Louis, MO, USA), SC-71 (anti-MHCIIa; Schiaffino et al., 1989), BF-F3 (anti-MHCIIb; Schiaffino et al., 1989) and BF-35 (recognizes all MHC isoforms except for MHCIId(x); Schiaffino et al., 1989). In order to detect developmental MHC isoforms, the following antibodies were used: NCL-d (anti-MHC embryonic) and NCL-n (anti-MHC neonatal) (Novocastra Laboratories, Newcastle, UK) and BF-45 (anti-MHC embryonic; Schiaffino et al., 1988). Secondary biotinylated horse anti-mouse IgG (rat-adsorbed and affinity-purified) and biotinylated goat anti-mouse IgM (used with BF-F3) were obtained from Vector Laboratories (Burlingame, CA, USA). Normal control mouse IgG was obtained from Santa Cruz Biochemical (CA, USA).

6.2.7. Immunohistochemical staining

10μm serial sections of fetal day 90 *semitendinosus* muscle were collected on poly-L-lysine coated slides (Electron Microscopy Sciences, Fort Washington, PA, USA) and stored at –80°C for later analysis. Adult *triceps brachii* tissue was also collected to confirm reactivity of the antibodies with porcine tissue. The avidin biotin peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) was used to visualise immunoreactivity. Frozen muscle sections were air-dried, washed once in phosphate-buffered saline with 0.1% (v/v) Tween 20 (PBS-Tween), twice in PBS, and incubated for 15 min in 3% (v/v) H_2O_2 in methanol. Sections were subsequently washed and incubated overnight at 4°C in the appropriate blocking solution (BS) containing 20% (v/v) Avidin D blocking solution (Vector Laboratories, Burlingame, CA, USA). (BS-1;

2.4% (w/v) protease-free BSA, 6% (v/v) Horse serum, and 0.1% (v/v) Tween 20 in PBS, pH 7.4. BS-2; as for BS-1 except goat serum was substituted for Horse serum). Sections were washed in PBS followed by incubation with the primary antibody for 1 h at room temperature in appropriate BS containing 20% (v/v) Biotin blocking solution (Vector Laboratories, Burlingame, CA, USA). Primary anti-mouse IgG monoclonal antibodies were diluted in BS-1 as follows: NOQ7.5.4D 1:4000; BA-D5 (culture supernatant) 1:100; F88.12F8 1:10; MY-32 (culture supernatant) 1:1000; SC-71 (culture supernatant) 1:50; BF-35 (purified IgG fraction) 0.1μg/ml; NCL-D 1:20; NCL-N 1:10 and BF-45 (culture supernatant) 1:1000. The IgM monoclonal antibody BF-F3 (culture supernatant) was diluted 1:50 in BS-2.

Control sections were processed in parallel incubations in which the primary antibody was omitted or substituted with non-specific control mouse IgG (Santa Cruz, CA, USA). Sections were washed as before and incubated for 1 h at room temperature with the appropriate secondary antibody (biotinylated horse anti-mouse IgG or biotinylated goat anti-mouse IgM (BF-F3) diluted 1:500 in BS-1 (IgG) or BS-2 (IgM)). Sections were then washed and incubated with Vectastain ABC reagent for 45 min at room temperature, washed again and reacted for 6 min with the substrate solution containing diaminobenzidine, H_2O_2, and $NiCl_2$ in 50 mmol/l Tris·HCl, pH 7.5 (Vector Laboratories DAB Substrate Kit for Peroxidase). The reaction was stopped by washing several times with distilled water. After dehydration in ethanol, sections were cleared with xylene and mounted with Entellan (Merck, Darmstadt, Germany).

Image acquisition was carried out using a motorised scanning stage Zeiss Axioplan IIM Universal Microscope (Carl Zeiss Jena GmbH, Jena, Germany) and a Photometrics CoolSNAP HQ Camera (Roper Scientific, Tucson, AZ, USA) in conjunction with the Metamorph Imaging System (Universal Imaging Corporation, Downingtown, PA, USA). Images of the entire cross section of each muscle were obtained and saved to CD in montage format for Metamorph Image analysis.

Serial sections of day 90 tissue stained for the various MHC isoforms were examined to determine which muscle fibre isoforms were present. Sections stained with the NOQ7.5.4D antibody (anti-MHCIβ) were used to determine *semitendinosus* muscle total cross-sectional area (CSA), muscle fibre type (i.e. primary or secondary), and fibre CSA. Two distinct areas of the muscle were examined. For each individual muscle, five fields were selected at random from the deep red portion and five random fields from the superficial white portion of the muscle. Results from both portions of the muscle were averaged, and the resulting 10 fields per muscle encompassed a total area of $1.48mm^2$. An average of 10,410 ± 981 fibres (mean ± SD) were examined per muscle. Mean CSA of primary and secondary fibres were measured from at least 300 fibres of each type. Total numbers of primary and secondary fibres were calculated for each muscle by extrapolating the mean number of fibres of each type per mm^2 to the actual CSA of the entire *semitendinosus* muscle determined by measurement using the Metamorph Imaging System. Two piglets were analysed per litter and the results were averaged within sow to examine the effects of uterine crowding on muscle fibre development.

6.2.8 Myosin extraction

Frozen muscles were pulverised and extracted in six volumes of a buffer containing 0.3 mol/l KCl, 5 mmol/l $MgCl_2$, 5 mmol/l EGTA, 100 mmol/l $Na_4P_2O_7$, 1 mmol/l dithiothreitol and 5mg/ml Complete Mini Protease Inhibitor (Roche Diagnostics GmBH, Mannheim, Germany), pH 8.5. The solution was stirred for 30 min on ice and cleared by centrifugation for 5 min at 12,000 x g at 4°C. The supernatants were diluted 1:1 (v/v) with glycerol and stored at -20°C. Protein concentrations were determined using the Bradford procedure (Bio-Rad Laboratories, Hercules, CA, USA).

6.2.9 Standard SDS-PAGE

The MHC complement of whole muscle extracts was analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a slightly modified version of the method described by Hämäläinen and Pette, (1996), which has been described elsewhere (Putman et al., 2003). Briefly, the separating gel contained 7% (w/v) polyacrylamide and 35% (v/v) glycerol, and the stacking gel was composed of 4%

(w/v) polyacrylamide and 25% (v/v) glycerol. The upper buffer (25 mmol/l Tris-base, 192 mmol/l glycine, 10 mmol/l SDS) was supplemented with 0.2% (v/v) 2-mercaptoethanol. Before loading, extracts were incubated for 5 min at 100°C in a buffer containing 2.3% (w/v) SDS, 8% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 500 mmol/l Tris-base (pH 7.2), 0.1% (w/v) bromophenol blue and 44% (w/v) sucrose. Samples were cleared by centrifugation at 12,000 x g at 4°C, and 0.75 μg of total protein was loaded in each well. MHC isoforms were separated at 10°C for 24 h at 275 V (constant voltage) and visualised by silver staining. The relative MHC isoform contents were quantified densitometrically using the Syngene Chemigenius gel documentation system and GeneTools gel analysis software (Syngene, Cambridge, UK). The scheme used for identifying the various MHC isoforms was validated by Western blotting and by comparison with rodent muscles.

6.2.10 Western blotting

Western blotting was carried out using the Unblot In-Gel Chemiluminescent Detection Kit for Biotinylated Antibody Probes (Pierce Biotechnology, Rockford, IL, USA). Following standard SDS-PAGE, gels were pretreated with 50% (v/v) isopropyl alcohol and then washed in ultrapure water. Gels were then reacted for 1 h at room temperature with monoclonal antibodies against various MHC isoforms: MF-20 (1:10), NOQ7.5.4D (1:7000), MY-32 (1:160,000), SC-71 (1:200), BF-35 (1:1000), NCL-D (1:40), NCL-n (1:20) and BF-45 (1:2000). Following incubation with the primary antibody, gels were washed in PBS containing 0.05% (v/v) Tween 20 (PBS-Tween 0.05%), and incubated for 1 h at room temperature with the appropriate biotinylated secondary antibody (biotinylated horse anti-mouse IgG or biotinylated goat anti-mouse IgM (BF-F3)). The gel was washed as before and then incubated in Streptavidin-HRP using a 1:500 dilution of the supplied kit reagent. The wash step was repeated, the gel was incubated for 5 min with Unblot substrate working solution (1:1 dilution of each kit component), and then washed in ultrapure water for 15 sec. All incubations were carried out with gentle shaking to ensure adequate exposure of the gel to each reagent. The gel was then placed between two cellophane sheets and exposed to a CCD camera to detect the chemiluminescent signal using the Syngene Chemigenius gel documentation system (Syngene, Cambridge, UK).

6.2.11 Statistical analysis

To determine the effects of treatment on ovulation rate, conception rate, number of viable embryos, embryonic survival rate, placental and embryo/fetal weights, placental efficiency, fetal organ weights, fetal brain:liver weight ratio, muscle weight and CSA, fibre number, secondary:primary fibre ratio and MHC isoform distribution, data were analysed as appropriate for a completely randomised design. Sow was used as the experimental unit for analysis, and fetal weights, placental weights, organ and muscle parameters were averaged within each reproductive tract (sow) before analysis.

Data were analysed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1990, SAS Inst. Inc., Cary, NC). The model included treatment group (CTR or LIG). Significance was considered as $P < 0.05$. The results are presented as means ± SEM.

Relevant associations within gestational age between number of viable embryos/fetuses, embryo/fetal weight, placental weight, placental efficiency, embryonic survival rate, plasma progesterone, fetal organ weights (day 90) and brain:liver weight ratio were examined using the INSIGHT procedure (SAS, 1990).

6.3 RESULTS

6.3.1 General results

Of the 60 sows slaughtered, 58 were confirmed pregnant by the presence of viable conceptuses. Removals from final analysis were due to missing data as a result of damage during tract removal and tissue collection (missing ovaries, etc). Overall ovulation rate for this sow population was 19.90 ± 0.36. The CTR sows had a lower ($P = 0.0537$) ovulation rate (19.23 ± 0.48) than the LIG sows (20.61 ± 0.50). Ovulation rates available for fertilisation differed between the two treatment groups ($P < 0.0001$; Table 6.1).

Both the number of viable embryos at day 30 and number of fetuses at day 90 were different between treatments ($P < 0.0001$; Table 6.1) and embryonic survival to day 30 and fetal survival to day 90 were both higher in the LIG group ($P < 0.05$; Table 6.1). Average placental weight was lighter in CTR animals at day 30 and day 90 of gestation ($P < 0.05$; Table 6.1). Average embryonic weight was not different between groups at day 30; however, average fetal weights at day 90 were lighter in CTR sows. Placental efficiency, calculated as the embryonic weight: placental weight ratio, was higher in the CTR group at day 30. However, this relationship was lost in the day 90 fetuses in which placental efficiency was not different between the two groups.

Average placental weight was positively associated with average embryonic weight at day 30 of gestation ($R^2 = 0.31$; $P < 0.01$; Figure 6.1a) and with average fetal weight at day 90 of gestation ($R^2 = 0.46$; $P < 0.01$; Figure 6.1b). Furthermore, average placental weight was negatively related to number of viable embryos at day 30 ($R^2 = 0.37$; $P < 0.01$; Figure 6.2a) and number of viable fetuses at day 90 ($R^2 = 0.45$; $P < 0.01$; Figure 6.2b). Although average embryonic weight was not associated with the number of viable embryos at day 30, a negative relationship was established between the number of fetuses and fetal weight at day 90 ($R^2 = 0.37$; $P < 0.01$; Figure 6.3).

Placental efficiency was not associated with average embryonic or fetal weight at either day 30 or day 90 of gestation, although it was weakly related to the number of viable embryos at day 30 ($R^2 = 0.14$; $P < 0.05$) and showed a trend towards significance with the number of viable fetuses at day 90 ($R^2 = 0.12$; $P = 0.06$). However, placental efficiency showed a strong relationship with average placental weight at day 30 ($R^2 = 0.42$; $P < 0.0001$) and day 90 ($R^2 = 0.56$; $P < 0.0001$).

6.3.2 Progesterone assay data

Sow plasma progesterone concentrations at 72 h after onset of standing heat ranged between 0.67 and 9.69 ng/ml. A positive association was observed between fetal survival rate to day 90 and progesterone concentration 72 h after onset of standing heat ($R^2 = 0.26$; $P < 0.01$; Figure 6.4b), but no such relationship was established for the day 30 sows ($P = 0.669$; Figure 6.4a).

6.3.3 Day 90 necropsy data

Fetal organ weight data at day 90 of gestation are shown in Table 6.2. The brain was the only organ for which there was no difference between treatments. All other organs were heavier in fetuses from LIG sows ($P < 0.05$). Furthermore, the brain:liver weight ratio was higher in the fetuses from crowded litters ($P < 0.01$), and the brain:*semitendinosus* muscle weight ratio and the brain:total number of secondary fibre ratio, were also higher in the CTR sows (both $P < 0.05$). The male:female ratio of the piglets chosen for necropsy (closest to the mean body weight of the litter) is shown in Tables 6.3 and 6.4. There were 14 male and 14 female piglets in the control group and 13 male and 15 female piglets in the ligated group.

Fetal weight was positively related to absolute weight of the fetal liver ($R^2 = 0.83$; $P < 0.0001$; Figure 6.5a), brain ($R^2 = 0.31$; $P < 0.002$; Figure 6.5b), heart ($R^2 = 0.83$; $P < 0.0001$; Figure 6.6a), lungs ($R^2 = 0.66$; $P < 0.0001$; Figure 6.6b), and spleen ($R^2 = 0.45$; $P < 0.0001$; Figure 6.6c). When relative organ weights were calculated as the absolute organ weight:body weight ratio, mean relative brain weight showed a strong negative association with mean fetal weight ($R^2 = 0.68$, $P < 0.0001$; Figure 6.7b). In contrast, mean relative liver weight was only weakly related to average fetal weight ($R^2 = 0.18$; $P > 0.05$; Figure 6.7a), and no associations were evident ($P \geq 0.1$) with mean relative heart, lung and spleen weights (Figure 6.8). The mean brain:liver weight ratio was negatively related to mean fetal weight ($R^2 = 0.58$; $P < 0.0001$; Figure 6.9a) and mean placental weight ($R^2 = 0.55$; $P < 0.0001$; Figure 6.9b), but positively related to the number of viable fetuses ($R^2 = 0.53$; $P < 0.0001$; Figure 6.10).

6.3.4 Immunohistochemical analysis

6.3.4.1 Myosin isoforms

Immunohistochemistry results are shown in Figure 6.11. For the day 90 fetal *semitendinosus* tissue, positive specific staining for MHCIβ (NOQ7.5.4D and BA-D5), and MHCIIa (SC-71) was observed. MY-32 (all fast MHC) seemed to give a positive reaction with primary fibres in addition to positively staining secondary muscle fibres in day 90 tissue. BF-35 (recognizing all MHC isoforms except for MHCIId(x)) resulted in staining of all fibres present, probably indicating that no IId(x) fibres are present at day 90. No staining was observed for F88.12F8 (specific to MHCIα; data not shown) or BF-F3 (specific to MHCIIb). Surprisingly, a negative result was also obtained for the developmental antibodies NCL-d (anti-MHC embryonic), NCL-n (anti-MHC neonatal) and BF-45 (anti-MHC embryonic). In adult *triceps brachii* tissue, which was used to confirm reactivity of the antibodies used with pig tissue, positive staining was observed using NOQ7.5.4D, BA-D5, and SC-71 and, in contrast to the day 90 *semitendinosus* tissue, BF-35 did not stain all fibres, indicating the presence of pure IId(x) fibres. Additionally, positive staining was observed for BF-F3, indicating that MHCIIb fibres are also present in adult tissue. F88.12F8 and MY-32 have previously been shown to react with swine MHCIα (Lefaucheur et al., 1995, 1997) and all fast MHC respectively (Fazarinc et al., 1995).

6.3.4.2 Fibre count data

Muscle fibre development data obtained from the muscle sections stained using the antibody specific to MHCIβ (NOQ7.5.4D) are shown in Table 6.3. Average fibre number per mm^2 was not different between groups for either primary or secondary fibres, nor was average fibre CSA. However, muscle weight and muscle CSA were greater ($P < 0.05$) in fetuses from LIG sows. As a consequence, the total number of secondary fibres across the whole muscle was also greater in the fetuses of LIG sows ($P < 0.05$). There was no difference between the LIG and the CTR fetuses in the number of primary fibres, ($P = 0.073$) or the secondary:primary fibre ratio ($P = 0.129$).

6.3.5 Standard SDS-PAGE

The MHC complement of whole muscle extracts of day 90 fetal tissue was analysed by SDS-PAGE and measured densitometrically. The electrophoretic separation of four different MHC isoforms is shown in Figure 6.12. A comparison of their mobilities with bands obtained from rat muscle, adult pig muscle and neonatal pig muscle (Figure 6.12) was carried out, and the distribution of MHC isoforms is shown in Table 6.4. The fetal MHC isoform was most abundant, followed by embryonic, type IIa and type Iβ. There were no differences in relative isoform distribution between fetuses from LIG and CTR sows (Table 6.4).

6.3.6 Western blotting

Western blotting with the MF-20 antibody that recognizes all MHC isoforms, confirmed that the four bands observed on the protein gels were all isoforms of MHC. Various antibodies were used to determine the identification of the four myosin bands. In the day 90 tissue, the band with the greatest mobility (band d) was identified as MHC Iβ based on reactivity with NOQ7.5.4D. The band with the second greatest mobility (band c) was identified as MHCIIa based on reactivity with SC-71 (Figure 6.13). Positive bands were not observed using the developmental antibodies NCL-d, NCL-n and BF-45 due to the apparent lack of reactivity of these antibodies with porcine muscle. However, the two upper bands observed in the day 90 samples were tentatively identified as fetal (band b) and embryonic (band a), based on the gel patterns established by Lefaucheur et al., (2001). As expected, these fetal and embryonic bands were not observed in adult tissue.

6.4 DISCUSSION

Royston et al. (1982) identified growth-retarded neonatal piglets as members of a discrete subpopulation of one or more individuals within a litter. In later studies, van der Lende et al. (1990) reported that within-litter weight distribution of piglets was already established by the end of the embryonic stage of gestation at day 35, and that the level of embryonic loss was linked to different weight distribution patterns within a litter. They proposed that a reduction in within-litter weight variation might be one consequence of selective embryonic mortality in the preimplantation period, as originally suggested by Pope (1994). Nevertheless, Hegarty and Allen (1978) showed that within a litter, runts have a reduced muscle growth potential and, as a consequence, needed 23 days longer to reach a weight of approximately 105kg. However, in the context of the present study, it is important to recognise that earlier studies on within-litter variation in development, and associations with embryonic loss, involved gilts with mean ovulation rates of around 14. In gilts and likely in weaned first-parity sows in existing commercial dam-line genotypes, ovulation rate and early embryonic survival are probably still the key determinants of litter size and, thus indirectly, of postnatal growth potential. However, evidence for changing patterns of prenatal loss discussed by Foxcroft (1997) suggests that in mature sow populations, pre-implantation selection among embryos may not be the most critical factor determining the pattern of pre- and postnatal development. In at least some of these sows, with markedly increased ovulation rates and low embryonic loss through the pre-implantation period, the number of conceptuses *in utero* around day 30 of gestation can greatly exceed uterine capacity. Based on existing literature, we hypothesised that this will have important consequences for subsequent development.

The specific objective of the present study was to investigate one experimental paradigm for testing the hypothesis that fetuses with differences in placental size, resulting from different levels of uterine crowding around day 30 of gestation, will have different developmental outcomes. Furthermore, rather than creating a distinct subpopulation of growth-retarded animals within a litter, preliminary evidence for a more universal effect of increasing numbers of conceptuses *in utero* on reduced placental size at day 28 to 30 of gestation (Almeida et al, 2000; Vonnahme et al, 2002), supported the suggestion that this might affect subsequent development of all surviving fetuses (Foxcroft, 1997).

Overall, ovulation rate for the third parity sow population studied was 19.90 ± 0.36, allowing the possibility of substantially increased uterine crowding compared to the gilts studied by van der Lende et al. (1990). However, this is still considerably lower than the ovulation rates of 26.9 ± 1.4, 26.6 ± 0.40 and 24.71 ± 0.38 observed in multiparous dam-line sows by Ozechowski (1998), Vonnahme et al. (2002) and in Chapter 4. Although CTR sows had a slightly lower ovulation rate than the LIG sows, the unilateral oviduct ligation procedure, resulted in an expected difference (19.2 vs 10.5) in the number of ovulated oocytes available for fertilization between the two treatments. This was associated with a difference in viable embryo number at day 30 (15.1 vs 9.3) and fetal number at day 90 (14.4 vs 9.4). Moreover, as the proportion of embryos surviving to day 30, and fetuses surviving to day 90, was higher in the LIG sows, it is likely that mechanisms driving selective reduction in the number of pre-implantation embryos, as well as mechanisms matching the number of conceptuses in the post-implantation period to functional uterine capacity, were both operative in the CTR sows. However, these sows showed only a modest level of crowding *in utero* at day 30.

Evidence supporting the concept of progesterone-dependent mechanisms mediating embryonic survival has been reviewed previously (Foxcroft, 1997). Although these associations have largely been reported in gilts subjected to nutritional manipulation, the positive correlation observed between 72 h plasma progesterone concentrations and fetal survival at day 90 provide support to previous evidence for an association between progesterone and prenatal survival in sows (Clowes et al., 1994; van den Brand et al., 2000).

Average placental weight was lighter in CTR animals at day 30 and consistent with earlier studies (Almeida et al., 2000; Vonnahme et al., 2002), average placental weight was negatively correlated with the number of viable embryos. Also consistent with previous data, while placental weight was affected at day 30, average embryo weight was not different between groups at this stage of gestation. However, at day 90 of gestation, both placental weight and fetal weight were lighter in CTR sows, and placental weight was positively correlated with fetal weight (Figure 6.1b). These relationships are consistent with the positive correlation between average placental weight at term and average birth weight seen in our previous studies (Town et al., 2002; $R^2 = 0.76$, $P < 0.001$), and by Biensen et al. (1999) and Wilson and Ford (2000). In the present study, both fetal weight and placental weight showed an inverse relationship with number of viable fetuses at day 90, in agreement with data on fetal weight reported by Bauer et al. (1998). Figure 6.2b shows the relationship between placental weight and average number of viable embryos at day 30. Although a linear regression was the best fit for the overall data, differences in relative crowding within the CTR animals resulted in little change in placental weight, suggesting that some minimal placental weight is probably needed for any conceptus to survive to the post-implantation period of gestation.

Collectively, we interpret these data as indicating that day 30 embryos are less sensitive to nutrient limitations than fetuses in later gestation. However, we still reasoned that, in the absence of placental compensation in terms of increased placental efficiency, the early limitation in placental size in CTR sows at day 30 would ultimately limit fetal development later in gestation. This was borne out by the day 90 data, in terms of decreased fetal and placental weights, and an increased brain:liver weight ratio.

Interestingly, although placental efficiency (calculated as the fetal weight:placental weight ratio, as suggested by Wilson et al., 1999) was higher in the CTR group at day 30, there was not a difference between the two groups at day 90. However, neither at day 30, nor day 90, was placental efficiency correlated with average embryonic or fetal weight. Furthermore, considering the weak association between placental efficiency and the number of viable embryos at day 30 and the number of viable fetuses at day 90, along with the strong negative correlations between placental efficiency and average placental weight at day 30 and day 90, fetal body weight appears to be more dependent on placental size, than placental efficiency. These data are consistent with the hypothesis that during late gestation, breed-specific mechanisms exist to maintain optimal fetal growth (Biensen et al., 1998). These authors reported that in the Meishan breed, fetal growth depends on an increase in placental vascular density, whereas York (white-line) fetuses, similar to those studied in the present experiment, rely on an increased surface area for increased placental exchange. Therefore, as limited placental size was expected to be a critical factor for later development in the CTR sows, data from the study of IUGR in other species, and particularly those involving the experimental restriction of placental function (as reviewed by McMillen et al., 2001), suggest that the relative uterine crowding seen *in utero* in the sows in the present study would affect the pattern of organ development. At day 90, the brain was the only organ for which there was no difference between treatments, whereas all other organs were heavier in the fetuses from LIG sows. These observations provide initial evidence of brain sparing effects, as uterine capacity became increasingly limiting for placental and subsequently fetal development in CTR sows.

The observed positive associations between absolute fetal organ weights and body weight were expected. Relative organ weights (organ:body weight ratio) at a fixed stage of gestation (day 90) were used to further investigate effects of IUGR on the pattern of organ development over the wide range of fetal body weights observed. This method allowed the identification of disproportionate changes of relative organ size with change of absolute body size, which occurs to the greatest extent in the brain.

Whilst relative heart, lung and spleen weights were unrelated to fetal weight, and relative liver weight was only weakly associated with fetal weight, relative brain weight showed a strong negative relationship to fetal weight (Figure 6.7b), consistent with classic brain-sparing effects. Finally, the brain:liver weight ratio has been used as perhaps the most definitive measure of intrauterine growth retardation (Bauer et al., 1998). The strong negative associations between brain:liver weight ratio and both fetal weight (Figure 6.9a) and placental weight (Figure 6.9b), the higher brain:liver weight ratio in fetuses from CTR litters, and the strong positive relationship between brain:liver weight ratio and the number of viable fetuses (Figure 6.10), defined the extent of detrimental effects of low fetal bodyweight and decreased placental size on prenatal development. Clearly, brain sparing (as indicated by a high brain:liver weight ratio), occurs to a greater extent in lower bodyweight animals. McMillen et al. (2001) have suggested that the maintenance of brain mass is of primary importance for all fetuses, and that whilst compensatory mechanisms are needed to maintain disproportionate brain growth in growth-restricted fetuses, similar physiological mechanisms must also operate, albeit to a lesser extent, to ensure that brain mass is maintained within an optimal range even in normal fetuses.

A specific focus on space-dependent effects of IUGR on muscle development is clearly relevant to meat-producing species like the pig. Therefore, evidence that the brain:semitendinosus muscle weight ratio, and the brain:total secondary muscle fibre ratio, were also higher in the CTR sows, is of considerable economic as well as physiological significance. A clearer understanding of the origin and extent of effects of uterine crowding on muscle fibre development is clearly important. The confirmation of appropriate immunohistochemical and gel electrophoresis technologies for identifying crowding effects on muscle fibre development was, therefore, an important initial step. Adult pig muscle (*triceps brachii*), and fetal *semitendinosus* muscle tissue from day 30 and 50 of gestation, were included in the analysis to allow comparison with day 90 tissue and to facilitate identification of MHC isoforms. As discussed by Lefaucheur et al. (2002), the use of monoclonal antibodies for immunocytochemistry on serial sections constitutes a powerful approach to typing myofibres. However, whilst most available specific antibodies raised against different MHCs have been successfully used in the rat (Schiaffino et al., 1989), their specificity in pig muscle remains to be documented.

In an extension of the methodology used by Lefaucheur et al. (2002), Western blotting was used in combination with immunohistochemistry in the present experiment to further elucidate the MHC isoforms present in porcine fetal muscle tissue. Western blotting with the MF-20 antibody that recognizes all MHC isoforms confirmed that the bands observed by silver staining were all isoforms of MHC. The isoform that displayed the greatest electrophoretic mobility was identified as MHCIβ, and was consistent with the findings of Lefaucheur et al. (2001). Using Western blotting 'In gel' detection (Figure 6.13), a single band was observed for all samples using MHCIβ. This result was also confirmed using immunohistochemistry, where positive reactivity was observed using BAD-5 (specific staining for MHCIβ) for both adult *triceps brachii* tissue and fetal day 90 *semitendinosus* tissue. NOQ7.5.4D also showed specific staining for MHCIβ in day 90 *semitendinosus* tissue (Figure 6.11). Confirming the results of Lefaucheur et al.

(2001), who examined newborn piglet tissue, none of the primary fibres reacted with the antibody F88.12F8 (specific to MHCIα) in day 90 *semitendinosus* tissue. This further indicates that MHCIα is not present in the *semitendinosus* muscle until after birth in the pig.

Using immunohistochemistry, positive staining was observed for MHCIIa (using SC-71) for both day 90 and adult tissue; expression of this isoform was confirmed using Western 'In gel' detection and corresponded to the band with the second greatest electrophoretic mobility. In contrast to Lefaucheur et al. (2001) who noted that all three adult MHCII isoforms (i.e. IIa, IIx and IIb) appeared to be co-migrating in porcine muscle samples using standard SDS-PAGE technique, three fast MHC isoforms were observed in adult tissue in the present study. The presence of four bands in the adult tissue samples is in agreement with the immunohistochemical results from the present study which specifically identified Iβ, IIa, IIb and IIx isoforms. In the case of day 90 fetal tissue, it is likely that band c (Figure 6.12) is solely MHCIIa, as there was no evidence for the presence of IIb or IIx fibres based on the immunohistochemical results.

The two upper bands observed in the day 90 samples by gel electrophoresis (Figure 6.12) were tentatively identified as fetal (band b) and embryonic (band a), in accordance with the results obtained by Lefaucheur et al. (2001). The anti-MHC fetal antibody (F88 4C10) used by that group was no longer commercially available to allow more direct confirmation of their results. However, the relative electrophoretic mobilities of these isoforms in the present study were identical to those reported by Lefaucher et al. (2001). Developmental antibodies NCL-d, NCL-n and BF-45 that have been used in studies of tissue from other species (NCL-d; Putman et al., 2000; NCL-n; Ecob-Prince et al., 1989; BF-45; Schiaffino et al., 1988; Putman et al., 2003) do not appear to recognise the corresponding porcine isoforms by either immunohistochemical or Western blotting methods. The MHC isoform distribution results for day 90 fetal samples are shown in Table 6.4. The fetal MHC isoform was most abundant, followed by embryonic, -IIa and -Iβ. From the perspective of effects of crowding *in utero* and IUGR on myogenesis, there were no differences in the patterns of isoform distribution between fetuses from LIG and

CTR sows, indicating the absence of qualitative differences in myogenesis. Furthermore, as there was no difference in either primary or secondary average fibre number per mm^2 nor in fibre CSA, and no difference in the number of primary fibres between the LIG and the CTR fetuses, the increase in average muscle weight and average muscle CSA in fetuses from LIG sows appeared to be dependent on a greater number of secondary muscle fibres. This latter observation does, however, indicate a quantitative reduction in myogenesis in the CTR group. The impacts of manipulations *in utero* on the performance of adult skeletal muscle in different species have been comprehensively reviewed by Maltin et al. (2001). The results of the present study extend the findings of previous work in the pig in which both naturally occurring IUGR within a litter (Aberle, 1984) and nutritional manipulation during gestation (Dwyer et al., 1994) preferentially affected secondary muscle fibre development.

In the context of muscle fibre development it is important to consider the critical period during gestation when muscle fibre differentiation occurs. All available evidence indicates that differentiation of primary and secondary muscle fibres will be completed by day 90 in the pig, as discussed in chapter 2. Therefore, even if the effects of uterine crowding on muscle fibre numbers are associated with decreases in fetal weight at day 90, subsequent increases in fetal weight will not correct the problem of limited muscle fibre development. The existence of limited periods or "critical windows" of time for cell multiplication and differentiation in different organs and tissues is an essential driver of detrimental long term effects of IUGR.

In summary, the present study indicates that even moderate crowding of the uterus in the early period of gestation affects fetal development of the surviving conceptuses in a manner analogous to IUGR. These results raise important questions for fetal and postnatal development, particularly with respect to the development of the fetal muscle fibres. In prolific sow genotypes with still higher ovulation rates, greater uterine crowding will exert even more severe effects on fetal muscle development and consequently on postnatal growth potential. These data therefore provide important insights into the biological basis of variability in postnatal growth performance that has become an important economic concern for the swine industry.

Table 6.1 Reproductive characteristics (means ± SEM) of control (CTR) and unilaterally oviduct-ligated (LIG) sows (N=58).

	Treatment group		
Parameter	**CTR (n=30)** **"Relatively Crowded"**	**LIG (n=29)** **"Non-Crowded"**	**P-value**
Overall ovulation rate	19.2 ± 0.5 (n = 30)	20.6 ± 0.5 (n = 28)	0.054
Ovulation rate available for fertilization	19.2 ± 0.5	10.5 ± 0.6	< 0.001
Number of sows at day 30	**n = 15**	**n = 15**	
Number of viable embryos (d30)	15.1 ± 0.8	9.3 ± 0.8	< 0.001
Embryonic survival to d30 (%)	79 ±3	91 ± 2	0.006
Average placental weight (g) at d30	19.2 ± 1.0	26.2 ± 1.4	< 0.001
Average embryo weight (g) at d30	1.15 ± 0.05	1.22 ± 0.05	0.34
Placental efficiency at d30	0.071 ± 0.005	0.050 ± 0.003	0.001
Number of sows at day 90	**n = 15**	**n = 14**	
Number of viable fetuses (d90)	14.4 ± 0.5	9.4 ± 0.7	< 0.001
Fetal survival to d90 (%)	76 ± 3	84 ± 3	0.029
Average placental weight (g) at d90	219 ± 8	274 ± 14	0.003
Average fetal weight (g) at d90	588 ± 18	679 ± 18	0.002
Placental efficiency at d90	2.84 ± 0.09	2.64 ± 0.12	0.20

P-values indicate the significance of the main effect of treatment.

Table 6.2 Average empty carcass and body organ weights and brain:organ weight ratios (means ± SEM) in fetuses from control (CTR) and unilaterally oviduct-ligated (LIG) sows (N=29) at day 90 of gestation.

	Treatment group		
Parameter	**CTR (n=15) "Relatively Crowded"**	**LIG (n=14) "Non-Crowded"**	**P-value**
Spleen (g)	0.84 ± 0.04	1.07 ± 0.09	0.015
Liver (g)	17.81 ± 0.63	21.48 ± 0.94	0.003
Heart (g)	4.09 ± 0.15	4.65 ± 0.15	0.013
Lungs (g)	17.55 ±0.64	19.61 ± 0.64	0.031
Kidneys (g)	6.33 ± 0.18	7.35 ± 0.25	0.002
Brain (g)	19.65 ± 0.33	20.02 ± 0.41	0.48
Empty carcass (g)	464.7 ± 13.5	536.4 ± 14.3	0.001
Brain:Liver wt ratio	1.17 ± 0.04	0.97 ± 0.04	0.002
Brain:ST Muscle wt ratio	10.49 ± 0.43	9.25 ± 0.33	0.031
Brain:Total secondary fibre ratio	$(62 \pm 3.5)^{10-5}$	$(52 \pm 2.3)^{10-5}$	0.021

P-values indicate the significance of the main effect of treatment.

Table 6.3 *Semitendinosus* muscle fibre development data (means ± SEM) for day 90 fetuses from control (CTR) and unilaterally oviduct-ligated (LIG) sows (N=28).

	Treatment group		
Parameter	**CTR (n=14)** **"Relatively crowded"** **Male:Female ratio** **14:14**	**LIG (n=14)** **"Non-Crowded"** **Male:Female ratio** **13:15**	**P-value**
Primary fibre no/mm^2	29.5 ± 1.5	25.8 ± 1.3	0.073
Primary fibre CSA (μm^2)	123.5 ± 5.6	130.4 ± 4.0	0.33
Secondary fibre no/mm^2	678.7 ± 16.5	673.3 ± 18.6	0.83
Secondary fibre CSA (μm^2)	23.1 ± 1.5	20.2 ± 0.5	0.072
ST Muscle weight (g)	1.25 ± 0.06	1.47 ± 0.09	0.014
ST Muscle CSA (mm^2)	47.71 ± 2.85	58.78 ± 2.65	0.009
Number total primary fibres	1394 ± 81	1480 ± 57	0.39
Number total secondary fibres	32,691 ± 2098	39,628 ± 2074	0.027
Secondary:Primary fibre ratio	24.01 ± 1.49	26.80 ± 0.06	0.13

P-values indicate the significance of the main effect of treatment.

Table 6.4 Myosin Heavy Chain Isoform distribution (mean % ± SEM) in day 90 fetal *semitendinosus* muscle from control (CTR) and unilaterally oviduct-ligated (LIG) sows (N=28).

	Treatment group		
Parameter	**CTR (n=14)** **"Relatively Crowded"** **Male:Female ratio 14:14**	**LIG (n=14)** **"Non-Crowded"** **Male:Female ratio 13:15**	**P-value**
Embryonic MHC (%)	23.30 ± 2.37	25.05 ± 1.18	0.52
Fetal MHC (%)	58.61 ± 2.46	55.46 ± 1.72	0.30
Type IIa MHC (%)	13.26 ± 2.34	12.66 ± 0.82	0.81
Type Iβ MHC (%)	6.45 ± 1.12	6.84 ± 1.14	0.81

P-values indicate the significance of the main effect of treatment.

Figure 6.1 Relationship between average placental weight and (a) average embryo weight at day 30 of gestation (placental weight = 0.24 + 18.90(embryo weight), R^2 = 0.31; P = 0.0014) and (b) average fetal weight at day 90 of gestation (placental weight = placental weight = -23.19 + 0.426(fetal weight), R^2 = 0.46; $P < 0.0001$) in LIG animals (◇) and CTR animals (◆).

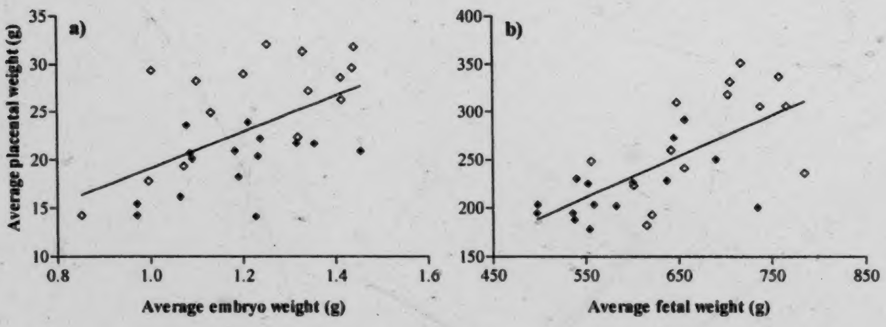


Figure 6.2 Relationship between average placental weight and (a) number of viable embryos at day 30 of gestation (placental weight = 32.85 + 0.83(number of embryos), R^2 = 0.37; P = 0.0003) and (b) number of viable fetuses at day 90 of gestation (placental weight = 368.34 – 10.19(number of fetuses), R^2 = 0.45; $P < 0.0001$) in LIG animals (◇) and CTR animals (◆).

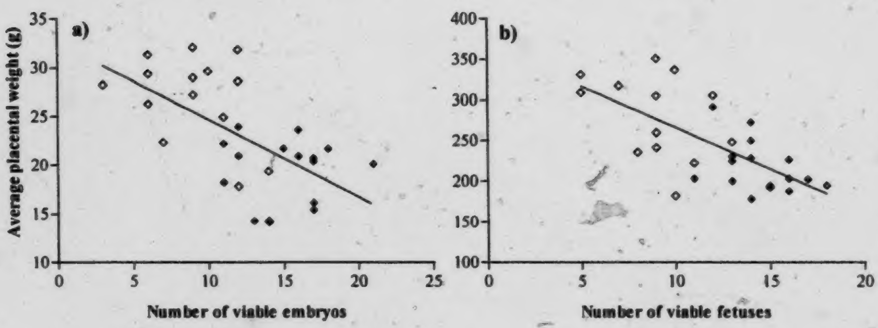


Figure 6.3 Relationship between average fetal weight and number of viable fetuses at day 90 of gestation (fetal weight = 807.45 – 14.59(number of fetuses), $R^2 = 0.37$; P = 0.0005) in LIG animals (◇) and CTR animals (◆).

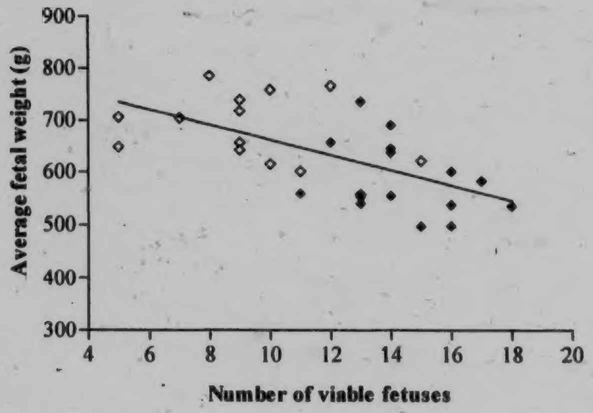


Figure 6.4 a) Non significant relationship between embryo survival rate at day 30 of gestation and sow plasma progesterone concentration (ng/ml) at 72h after onset of Standing Heat gestation (P = 0.669; n = 28 sows). b) Positive correlation between fetal survival rate at day 90 of gestation and sow plasma progesterone concentration (ng/ml) at 72h after onset of Standing Heat gestation (embryo survival to day 90 = 0.67 + 0.032(progesterone concentration), R^2 = 0.26; P = 0.008; n = 26 sows) in LIG animals (◇) and CTR animals (◆).

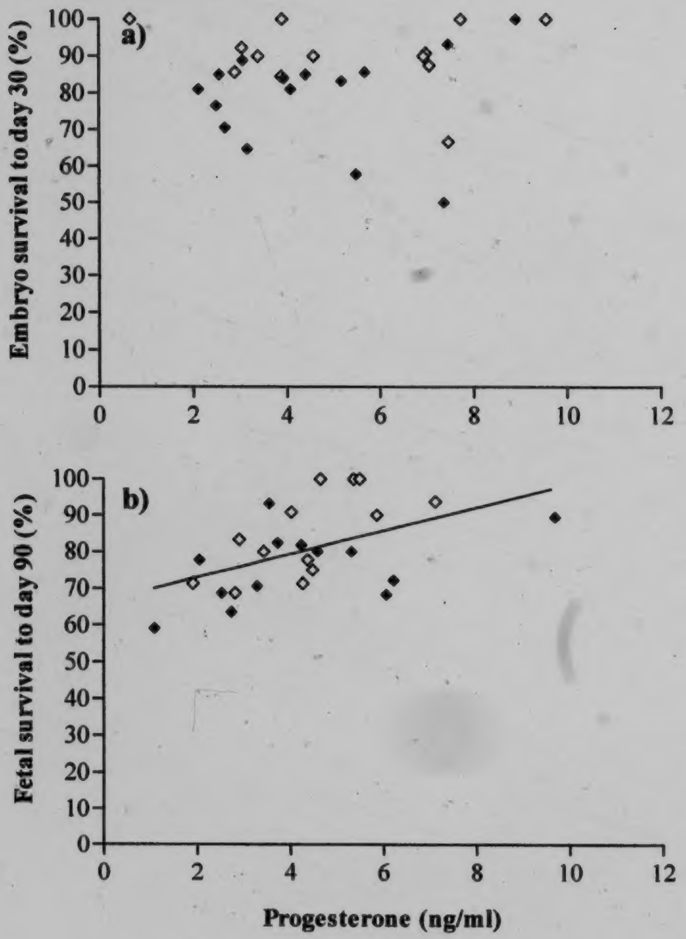


Figure 6.5 Relationship between (a) mean absolute liver weight (liver weight = -4.73 + 0.038(fetal weight), $R^2 = 0.83$; $P < 0.0001$) and (b) mean absolute brain weight (brain weight = 13.89 + 0.009(fetal weight), $R^2 = 0.31$; $P = 0.0017$) and average fetal weight at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

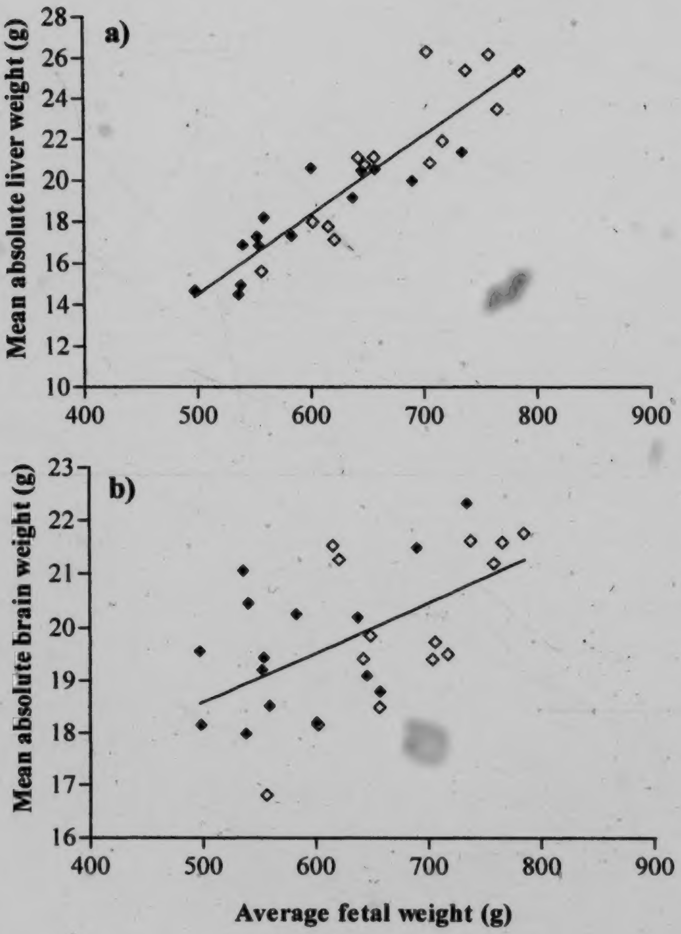


Figure 6.6 Relationship between average fetal weight at day 90 of gestation and a) mean absolute heart weight (heart weight = -0.023 + 0.007(fetal weight), $R^2 = 0.83$; $P < 0.0001$), b) mean absolute lung weight (lung weight = 2.36 + 0.026(fetal weight), $R^2 = 0.66$; $P < 0.0001$) and c) mean absolute spleen weight (spleen weight = -0.400 + 0.002(fetal weight), $R^2 = 0.45$; $P < 0.0001$) in LIG animals (◇) and CTR animals (◆).

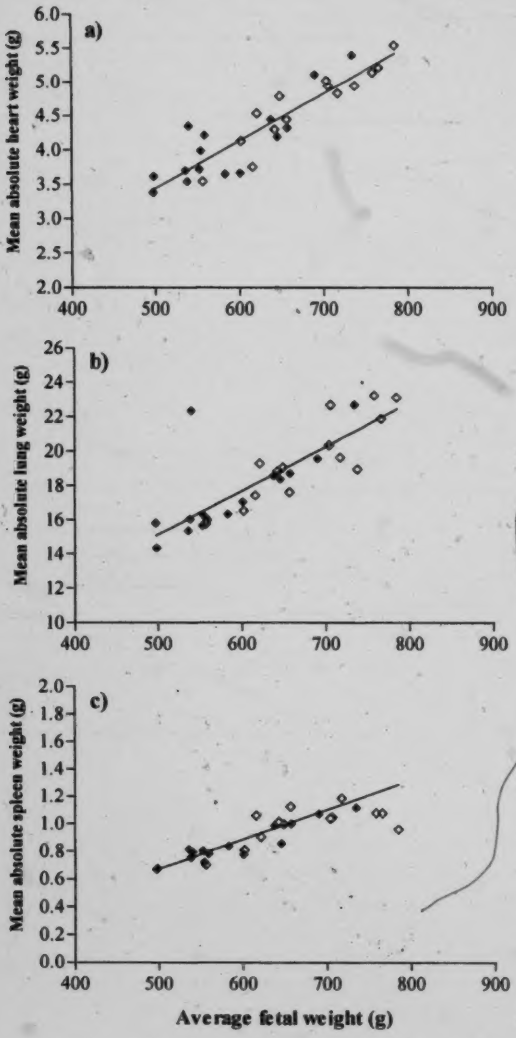


Figure 6.7 Relationship between (a) mean relative liver weight (relative liver weight = 0.023 + 1.2×10^{-5}(fetal weight), $R^2 = 0.18$; $P = 0.0231$) and (b) mean relative brain weight (relative brain weight = 0.054 – 3.6×10^{-5}(fetal weight), $R^2 = 0.68$; $P < 0.0001$) and average fetal weight at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

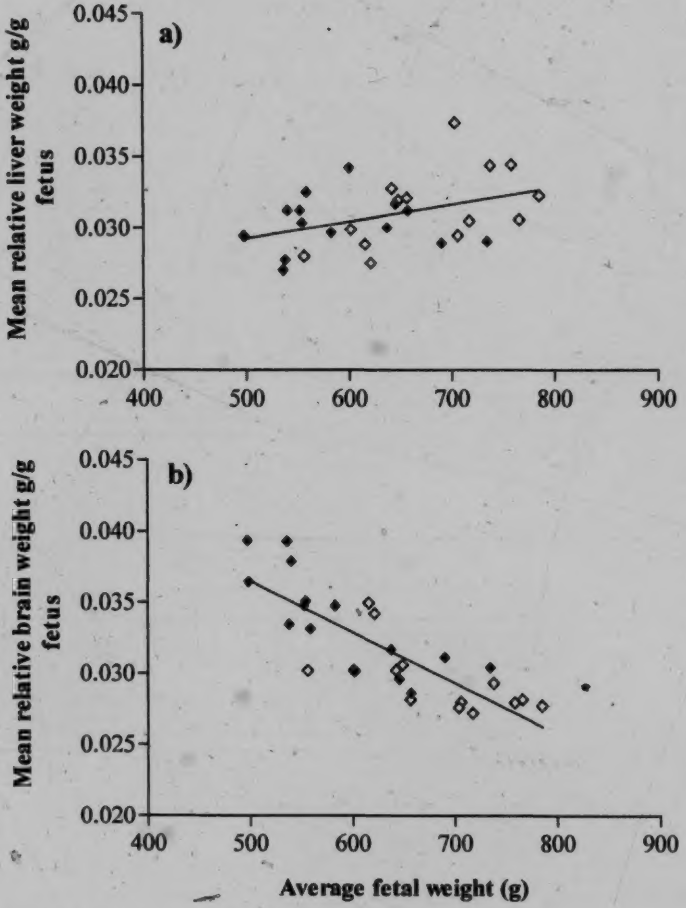


Figure 6.8 Absence of a relationship between mean fetal weight and mean relative weight of a) fetal heart (P = 0.94), b) lungs (P = 0.28), and c) spleen (P = 0.11) at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

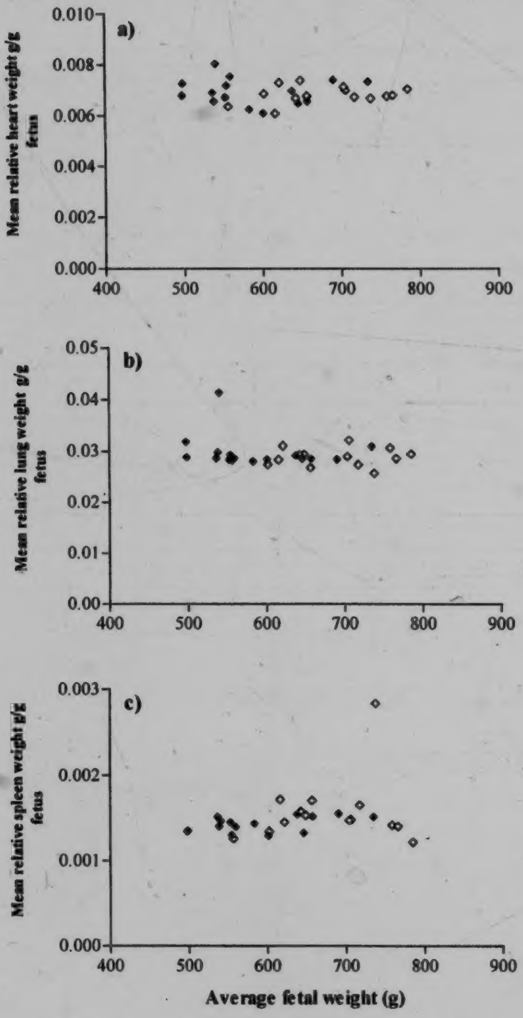


Figure 6.9 Relationship between mean brain:liver weight ratio and (a) average fetal weight (brain:liver = 2.14 – 0.002(fetal weight), $R^2 = 0.58$; $P < 0.0001$) and (b) average placental weight (brain:liver = 1.72 – 0.003(placental weight), $R^2 = 0.55$; $P < 0.0001$) at day 90 of gestation in LIG animals (◇) and CTR animals (◆).

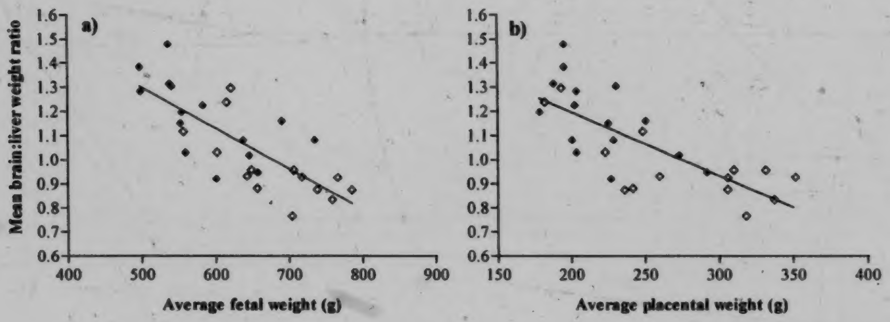


Figure 6.10 Relationship between mean brain:liver weight ratio and number of viable fetuses at day 90 of gestation (brain:liver = 0.61 + 0.039(number of fetuses), $R^2 = 0.53$; $P < 0.0001$) in LIG animals (◇) and CTR animals (◆).

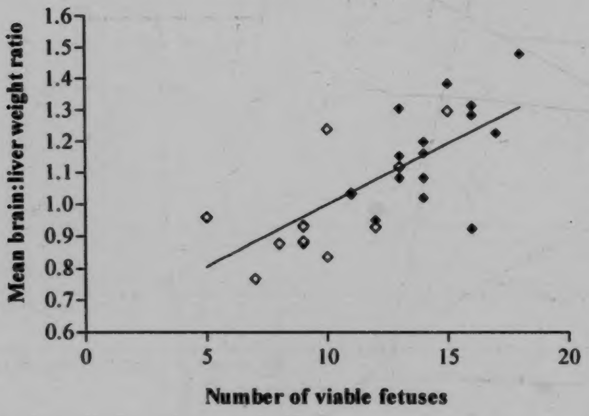


Figure 6.11 Immunohistochemical staining of the *triceps brachii* muscle of an adult pig (a-e) and the *semitendinosus* muscle of a d90 fetus (f-l). a) BA-D5 (anti MHCIβ); b) SC-71 (anti-MHCIIa); c) BF-F3 (anti-MHCIIb); d) BF-35 (anti MHC – except IIx/d); e) Ig G Control. Numbers denote the same fibres across serial sections. f) NOQ7.5.4D (anti-MHCIβ); g) BA-D5 (anti-MHCIβ); h) MY-32 (anti-MHCII all); i) SC-71 (anti-MHCIIa); j) BF-F3 (anti-MHCIIb); k) BF-35; l) Ig G Control. Scale bars represent 50 μm.

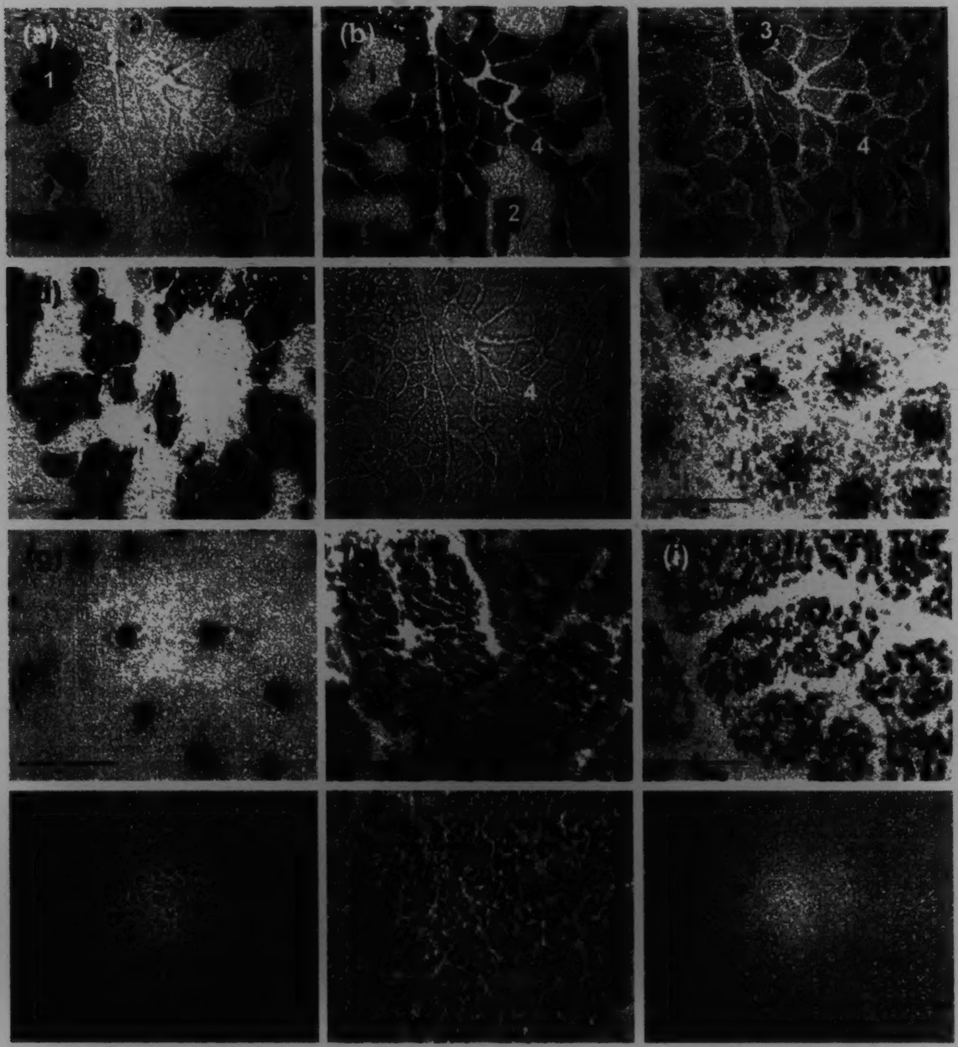


Figure 6.12 Electrophoretic separation of the MHC isoforms in rat *EDL/Soleus* muscle, adult pig *Triceps brachii* muscle, neonatal *Semitendinosus* muscle and day 90 fetal *Semitendinosus* muscle tissue.

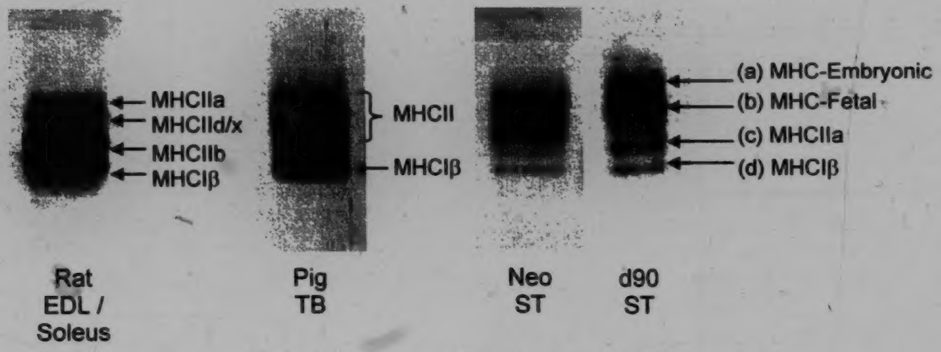
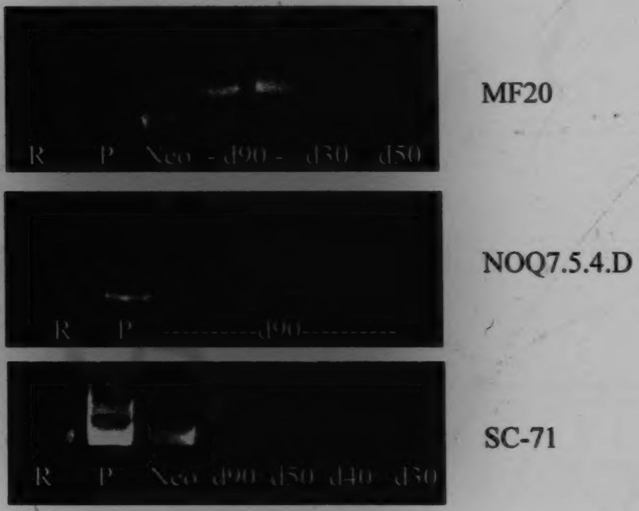

MHCIIa
MHCIId/x
MHCIIb
MHCIβ
MHCII
MHCIβ
(a) MHC-Embryonic
(b) MHC-Fetal
(c) MHCIIa
(d) MHCIβ
Rat EDL / Soleus
Pig TB
Neo ST
d90 ST

Figure 6.13 Western blot results for antibodies, MF20 (all myosin), NOQ7.5.4D (anti-MHCIβ) and SC-71 (anti-MHCIIa), using rat *EDL/Gastroc* muscle (R), adult pig *triceps brachii* muscle (P), neonatal porcine *semitendinosus* muscle (Neo) and fetal pig *semitendinosus* muscle from day 90, 50, 40 or 30 of gestation (d90, d50, d40, d30).


R P Neo - d90 - d30 d50
MF20
R P ----------d90----------
NOQ7.5.4.D
R P Neo d90 d50 d40 d30
SC-71

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CHAPTER SEVEN

GENERAL DISCUSSION

The largest proportion of prenatal loss in the pig has traditionally been viewed as occurring pre-implantation (as reviewed by Ashworth and Pickard, 1998). However, as described in Chapter 1, evidence suggesting that the dynamics of prenatal loss may be changing in some commercial dam-lines (Foxcroft, 1997) provided the impetus for the studies undertaken in this thesis. As evidenced by the observations of Almeida et al. (2000) and Vonnahme et al. (2002), an increasing number of embryos survive the implantation process, surpassing uterine capacity in the initial stages of pregnancy. However, uterine capacity ultimately exerts its effects and a proportion of embryos subsequently die between days 30 and 50 of gestation (Vonnahme et al., 2002). The potential for early crowding of the uterus would be of particular concern in situations where increased embryonic survival into the post-implantation stages of gestation occurs in combination with high ovulation rates reported in some populations of commercial dam-line sows (Orzechowski, 1998; Almeida et al., 2000; Vonnahme et al., 2002).

Placental size around day 30 has been shown to be negatively correlated with number of embryos by both Almeida et al. (2000) and Vonnahme et al. (2002). Yet as discussed extensively in Chapter 2, adequate placental function is vital for normal growth and development of the fetus. If placental compensatory mechanisms are not adequate, crowding of the uterus in the early post-implantation period of gestation could affect fetal development of surviving conceptuses, in a manner analogous to IUGR, raising important questions for both pre- and postnatal development. In the context of commercial grow-finish performance, the development of fetal muscle fibres, which start to differentiate during this early period of gestation (primary fibres begin to appear by day 35; Wigmore and Stickland, 1983), is of particular interest. Earlier studies of effects of maternal feed restriction on muscle development in the pig established lasting effects of reduced secondary muscle fibre numbers on postnatal growth (Dwyer et al., 1994). Therefore, the overall objective of these studies was to determine whether this changing pattern of embryonic loss affects placental and fetal development, with a particular focus on the development of skeletal muscle.

The initial study described in Chapter 3 used midline laparotomy at day 30 of gestation to determine embryo number and ovulation rate in bred gilts. At farrowing, piglets and placentae were matched using an umbilical tagging procedure and weighed. A relatively low mean ovulation rate (15.6 ± 0.6) and relatively high embryonic loss (34.8 %), likely as a result of an outbreak of Porcine Circovirus, meant that extremes of embryonic crowding at day 30 did not occur in this population of animals. A strong positive correlation between average placental weight at term and average birth weight was observed, although neither placental weight, nor birth weight showed a strong inverse relationship to litter size at term, initially suggesting that uterine capacity had only a moderate effect on intrauterine development in these animals. However, after piglet necropsy and removal of internal organs, the measurements of empty carcass weights were moderately and negatively correlated with litter size at term. A lack of association between placental efficiency and litter size, and a strong negative relationship between placental efficiency and placental weight, suggested that piglet birth weight was more dependent on placental size, than on placental efficiency. This conclusion is consistent with the hypothesis (Biensen et al., 1998) that during late gestation, breed-specific mechanisms exist, which maintain optimal fetal growth. In the Meishan breed which is characterized by increased litter size born, fetal growth depends on an increase in placental vascular density; in contrast in York (white-line) sows, fetuses rely on an increase in surface area in late gestation to increase placental exchange.

A significant inverse relationship was found between the mean relative mass of the fetal brain and the mean fetal body weight, indicating the effect of "brain sparing". Furthermore, a negative correlation between brain:liver weight ratio and mean piglet birth weight, and mean placental weight, clearly demonstrated the detrimental effects of low birth weight and decreased placental size on fetal development. Additionally, brain:liver weight ratio was positively correlated with litter size at term. Clearly, as reported previously (Bauer et al., 1998; McMillen et al., 2001), brain sparing (as indicated by a high brain:liver weight ratio) occurs to a greater extent in lower birth weight animals. However, consideration of the data presented in Chapter 3, shows that even in a relatively "uncrowded" uterus, evidence for brain sparing was still evident. Muscle fibre number was not examined in the study presented in Chapter 3, however, MHC isoform distribution was analyzed and will be discussed later in this chapter.

Following the study described in Chapter 3, the depopulation of a breeding unit within Swine Graphics Enterprises, Inc., (Webster City, Iowa, USA) presented a rare opportunity to obtain further data from a large population of commercial dam-line sows. We used the opportunity to work in collaboration with Dr Gene Gourley to examine relationships among ovulation rate, the pattern of prenatal loss, and placental and fetal development, and the effect of parity on the parameters of interest. Ovulation rate (22.7 ± 0.2 overall) was higher in P2/3 (23.6 ± 0.4) and P4+ (24.7 ± 0.4) than in G/P1 (20.2 ± 0.5). In all parities, ovulation rate was never a limiting factor for potential litter size born. However, low embryonic/fetal survival in this population of animals (61.8 ± 2.1% embryo survival rate at day 20-30), probably reflected the relatively poor health status of the herds at the time of depopulation and was similar to results observed by Vonnahme et al. (2002) in a comparable Swine Graphics Enterprises sow population. As a result, excessive crowding of embryos *in utero* at day 20-30 was not universally present in the sows included in this study. Furthermore, although embryonic weight was positively correlated with placental weight, in contrast to the data of Vonnahme et al. (2002) placental weight was not correlated with the number of viable embryos at day 20 to 30.

The number of surviving conceptuses was higher in the P2/3 sows than in other parity groups and an interesting parity by gestation day interaction was observed, whereby placental weights for P4+ were less than for P2/3 at day 20 to 30, whereas at day 85 to 90 the reverse situation existed. Collectively these results suggest that the increased number of viable embryos in parity group 2-3 may be due to an improved uterine environment and improved placental efficiency, particularly given the equivalent fetal weights observed in this group but a significantly lower placental weight at day 90. In contrast, despite maximal ovulation rates and comparable levels of pre-implantation survival in the parity 4+ sows, it appeared that the quality of the uterine environment and placental efficiency became limiting factors in later stages of gestation and reduced the functional "capacity" of the uterus.

The mean brain:liver weight ratio was negatively correlated with mean fetal weight and mean placental weight, as previously observed in the data from neonatal piglets described in Chapter 3. Parity also affected brain:liver weight ratio which was highest in the G/P1 group and lowest in P2/3 animals. The increase in relative brain mass with decreasing fetal weight was assumed to reflect a brain sparing effect. However, consistent with the conclusions made earlier, disproportionate brain growth in growth-restricted fetuses was apparent even when excessive *in utero* crowding of developing fetuses was not present.

The low ovulation rates observed in the gilts used in the first experiment, and the parity effect on ovulation rate seen in the depopulation study described in Chapter 4, led us to evaluate the use of multiparous sows as a population of choice in the development of an appropriate experimental model for the study of effect on *in utero* crowding on fetal development. Use of high health status animals at the University of Alberta Swine Research and Technology Centre, was also expected to be associated with higher embryo survival rates than those observed in the herd depopulation studies.

Chapter 5 described an experiment using weaned multiparous (parity 4-6), purebred sows, which either underwent embryo count surgeries at day 30 of gestation, or were subjected to unilateral oviduct ligation before breeding, and were then slaughtered around day 90 of gestation. Unfortunately from an experimental perspective, mean ovulation rate for this multiparous sow population studied was only 18.1 ± 0.46. Although this allowed the possibility of substantially increased uterine crowding compared to the gilts studied in Chapter 3, this ovulation rate is still considerably lower than the ovulation rates of 26.9, 26.6 and 24.7 observed in the multiparous dam-line sows studied by Orzechowski (1998), Vonnahme et al. (2002) and in Chapter 4, respectively. Oviduct ligation reduced ovulation rate available for fertilization from 18.5 ± 0.7 in the embryo count group to 10.2 ± 1.0 in the ligated group. Although embryonic survival to day 30 was not affected by oviduct ligation, fetal survival to day 90 was 21% higher in the ligated group compared to control sows, consistent with observations from Chapter 6. Whilst surgical intervention at day 30 may have decreased survival to day 90, embryonic survival at day 30 (in the subset of control animals) was 74%. This is again consistent with results of Chapter 6 and suggests that reproductive tract capacity was already exerting a selection effect even with only 13.7 ± 0.6 viable embryos *in utero* at day 30, and 11.5 ± 0.7 fetuses at day 90. The embryo survival rates of the ligated animals (83.6 ± 5.8%) in this population were clearly indicative of animals in good reproductive health.

Both the analysis of main treatment effects and the analysis of the association between the numbers of embryos or fetuses and placental weight, indicated a lack of an effect of number of embryos *in utero* on placental development in both control and ligated animals. This was in contrast to previous data (Almeida et al., 2000; Vonnahme et al., 2002; Chapter 6). Although this again limited the value of this experimental model, the data help to identify the threshold above which the number of offspring *in utero* created negative effects on placental growth. Additionally, there was no difference in placental efficiency between treatment groups in Chapter 5 at either day 30 or day 90, which suggested that a change in placental efficiency may be a compensatory mechanism that only exists when crowding detrimentally affects placental growth.

Interestingly, placental efficiency was seen to be lower in the purebred 4-6 parity sows examined in Chapter 5 compared to the crossbred parity 4+ sows in Chapter 4. Although the number of viable conceptuses and placental size at day 90 was seen to be very similar (11.5 vs 11.05 conceptuses and 255g vs 235g) in purebreds and crossbred sows, respectively, fetal weight was lower in crossbred sows compared to purebreds (599g vs 739g respectively) resulting in a lower placental efficiency in these purebred animals.

Given the lack of treatment effects on placental development, the lack of significant effects on embryonic weight at day 30 or fetal weight at day 90, brain:liver weight ratio or in ST muscle weight between groups, was not unexpected. Therefore, a fairly modest ovulation rate, coupled with a high embryo survival rate, and no effects of crowding on fetal development seen in this study are characteristics of sows at peak reproductive performance in terms of functional uterine capacity, measured by both the number and development of fetuses *in utero*.

A shortage of available animals led to a more limited allocation of multiparous sows to the experiment described in Chapter 5. However, since the oviduct ligation surgery had proved to be a successful model to reduce the number of embryos *in utero* in order to produce a "Non-Crowded" environment, a final experiment was carried out to investigate the effect of embryo number on muscle fibre development using more balanced treatment groups. Third parity F1 sows were used with the expectation that they would have higher ovulation rates than the purebred sows used in Chapter 5.

The study described in Chapter 6 involved unmodified, third parity, control sows and sows subjected to unilateral oviduct ligation prior to breeding. Sows slaughtered at either day 30 or 90 of gestation, were used to determine the effects of numbers of conceptuses *in utero* on prenatal, and particularly muscle fibre, development. Overall, ovulation rate for this population was 19.90 ± 0.36, again allowing the possibility of substantially increased uterine crowding compared to the gilts studied in Chapter 3. However, similar to Chapter 5, this was still considerably lower than the ovulation rates observed in multiparous dam-line sows by Vonnahme et al. (2002) and in Chapter 4. As in Chapter 5, oviduct ligation reduced the mean ovulation rate available for fertilization between the two treatments from 19.2 in control sows to 10.5 in ligated animals and this was associated with a difference in viable embryo number at day 30 (15.1 vs 9.3) and fetal number at day 90 (14.4 vs. 9.4). Moreover, embryonic survival to day 30, and fetal survival to day 90, were also higher in the LIG sows. These data suggest that mechanisms driving selective reduction in the number of pre-implantation embryos, as well as mechanisms matching the number of conceptuses in the post-implantation period to functional uterine capacity, were both operative in the CTR sows. Yet these sows showed only a modest level of crowding *in utero* at day 30.

In contrast to the study with purebred sows described in Chapter 5, placental weight at day 30 and 90, and fetal weight at day 90, were lower in control sows. Furthermore, the brain:liver weight ratio was higher in fetuses from control sows, indicative of brain sparing and IUGR. An important component of this brain-sparing effect was the lower muscle weight, muscle CSA and the total number of secondary fibres in control sow fetuses, although the number of primary fibres, and the secondary:primary muscle fibre ratio, did not differ between groups. Thus, even the relatively modest uterine crowding occurring naturally in CTR sows, negatively affected placental and fetal development, and the number of secondary muscle fibres.

Overall, data to support our hypothesis that changing patterns of prenatal loss exert negative effects on placental and fetal development were equivocal, due in part to an absence of high ovulation rates in the current study population. Although oviduct ligation surgery was an effective technique to *reduce* the potential number of embryos *in utero* at day 30 in order to establish "non-crowded" treatment groups, the Genex dam-line used at the University of Alberta did not exhibit the higher ovulation rates observed in other populations examined by Orzechowski (1998) and Vonnahme et al (2002) and in Chapter 4. Therefore, the extremes in terms of the number of embryos at day 30 were not seen in the current studies. The low ovulation rate observed in Chapter 5 (approximately 18) may have related to the purebred status of the sows. However, in Chapter 6, a mean ovulation rate of only 19.2 was observed in third parity F1 sows derived from the purebred sows used Chapter 5 and we conclude that a more modest ovulation rate may be characteristic of this particular commercial dam-line.

The second issue, which limited the impact of the experimental model we established, was low embryonic survival. In Chapter 3, embryonic survival rate in gilts (65.2 ± 4.9%) may have been due to an outbreak of Circovirus. In Chapter 4, where sows had the potential to show extremes of uterine crowding due to ovulation rates of up to 24.7 ± 0.4, low embryonic survival (61.8 ± 2.1%) at day 30, was also likely due to the low health status of the herd depopulated. Low embryonic survival was less of an issue for the sows studied in Chapters 5 and 6, however lower ovulation rates had already removed the possibility of obtaining the high extremes of embryonic number. Collectively, even the limited data available suggest important differences in key reproductive characteristics in existing commercial dam-line sows, presumably arising as an indirect response to selection for litter size.

The concept of uterine capacity has been widely studied, as discussed in Chapter 2. Differences in levels of prenatal loss between animals of different parities as observed in Chapter 4, suggested that the quality of the uterine environment and placental efficiency differs with age of the animal, altering the "functional capacity" of the uterus. In the gilt population of Chapter 3, despite the relatively low ovulation and embryonic survival rates, uterine capacity was clearly more limiting, since a moderate effect on birth weight, and brain sparing effects in surviving neonates, were observed that could have contributed to differences in postnatal growth performance. Similarly, in the third parity F1 sows studied in Chapter 6, even relatively modest uterine crowding produced negative effects on placental and fetal development, including a reduction in muscle size and in the number of secondary muscle fibres in day 90 fetuses. Overall, therefore, although uterine capacity has usually been discussed in terms of absolute numbers of embryos, our data suggest that a consideration of uterine capacity in terms of an ability to produce healthy offspring with the full growth potential may be equally important. A limit of 14 embryos (as defined by Dziuk, 1968) may not have to be reached before intrauterine crowding exerts its effects on fetal quality.

This raises questions about the thresholds for uterine crowding to exert an effect on fetal and placental development and whether these effects are likely to impair postnatal growth potential? In terms of practical implications for pork production, defining the thresholds for effects of uterine crowding is a complex issue. Collectively, results of the four studies presented in this thesis indicate that the potential for uterine crowding to impact fetal development depends on an interaction between parity, genotype and the health status of the sow population, and that increased numbers of embryos are dependent on ovulation rate, embryo survival rate and functional uterine capacity. Table 7.1 summarises various reproductive parameters of the control animals in each of the experiments in Chapters 3, 5 and 6.

Table 7.1 Reproductive parameters of control animals from three experimental studies.

Parameter (Means ± SEM)	**Chapter 3 Gilts (n = 23)**	**Chapter 5 Parity 4-6 (n = 11)**	**Chapter 6 Parity 3 (n = 30)**
Ovulation rate	15.6 ± 0.6	18.1 ± 0.46	19.2 ± 0.5^a
Day 30 embryo survival rate, %	65.2 ± 4.9	74.1 ± 3.0^a	79.0 ± 3.0^a
Number of embryos at day 30	10.0 ± 0.8	13.7 ± 0.6	15.1 ± 0.8^a
Litter size at day 90/term	8.5 ± 0.7	11.5 ± 0.7	14.4 ± 0.5^a
Effect on placental weight	No	No	Yes
Effect on fetal weight	Minimal	No	Yes
Effect on organ development (Brain:liver weight)	Yes	No	Yes

In Chapter 3, despite the fact that placental weight was not correlated with litter size, and body weight showed only a weak correlation with litter size at term, brain:liver weight was affected by an increase in litter size. Gilts clearly have a smaller uterine capacity than higher parity sows, although lower ovulation rates reduce the potential for developmental problems caused by uterine crowding. In these gilts, 10 embryos at day 30 seemed to be the threshold above which more detrimental effects on fetal and placental development would be observed. In Chapter 5, lower ovulation rates coupled with an increased uterine capacity, prevented the threshold for an effect of uterine crowding on fetal and placental development from being reached in parity 4-6 animals. In these mature sows, less than 14 embryos present at day 30 would likely not cause negative effects on development. From the current data, it appears that the highest risk group for detrimental developmental effects of increased embryo number appears to be the mid parity sows examined in Chapter 6. Increased ovulation and embryo survival rates coupled with an increased uterine capacity compared to the gilt population resulted in measurable effects on body organ and skeletal muscle development. In the extreme situations, developmental limitations will likely be associated with low birth weights, in addition to effects on organ and muscle development.

As discussed in Chapter 2, a common finding of IUGR is that the brain is the organ least affected by growth retardation, and disproportionate brain growth or "brain sparing" is maintained at the at the expense of other body organs. As suggested by McMillen et al. (2001) the maintenance of brain mass appears to be of primary importance for the survival of all fetuses, whether they are of normal birth weight or growth-restricted. McMillen et al. (2001) observed a "threshold effect" in the pattern of fetal kidney growth in sheep, whereby growth of the fetal kidney occurred in proportion to body weight until fetal body weight decreased below about 2 kg, at which point kidney mass was then maintained disproportionate to fetal body weight. If such relationships exist in the pig, they were not observed in our data. This was possibly due to the large variation in organ measurements and the fact that the organ weight measurements used were the mean of two representative piglets per litter.

As discussed in Chapter 2, there have been several definitions of inadequately grown fetuses in a litter. Generally fetuses or neonates weighing less than two standard deviations below the mean body weight for gestational age, or more frequently of the mean body weight of the relevant study population, is used to define IUGR in animal studies. However, fetuses weighing less than the 10th percentile (as in human clinical studies) was used to define IUGR by Bauer et al. (1998) and the brain:liver weight ratio increased more than 2.5 times in those newborn IUGR piglets (from approximately 0.7 in normal weight animals to 1.8 in cases of IUGR). In a subsequent study, Bauer et al. (2002) found that the brain:liver weight ratio increased from 0.98 ± 0.16 to 1.66 ± 0.22 in IUGR piglets. In the study described in Chapter 6 of this thesis, an increase in day 90 brain:liver weight ratio from 0.97 ± 0.04 in LIG fetuses to 1.17 ± 0.04 in CTR fetuses, although not as large an increase as those observed by Bauer and colleagues, was associated with a significant decrease in secondary fibre numbers in CTR fetuses.

Whilst statistical definitions of IUGR are useful to classify individuals in order to detect statistical differences between experimental groups of animals, the effects of IUGR are likely more complex and exist as a gradient of effects of growth restriction.

Furthermore, in terms of the extremes of IUGR that might exist, it is important to remember that the fetuses and neonates chosen for measurement in all studies would not have represented the extremes of IUGR, and effects of IUGR were likely to be expressed to an even greater extent in a significant proportion of the litter.

In addition, it is important to highlight that litter averages have been used to examine parameters of interest in the studies described in this thesis. Clearly, over the entire range of data from these study populations, a variable percentage of animals will fall into the high risk category for detrimental effects on growth and development, in terms of higher numbers of conceptuses *in utero*, resulting in higher brain:liver weight ratios. It is this range of variation in growth performance that is such an important concern for the swine industry.

The existence of limited periods or "critical windows" of time for cell multiplication and differentiation in different organs and tissues is an essential driver of detrimental long-term effects of IUGR. It is generally understood that a growth retarding stress during the phase of cell proliferation in an organ, will cause permanent restriction of cell population, while a similar stress during the later growth phase may cause a reversible impairment in cell size. This is particularly relevant in the context of muscle fibre development. All available evidence indicates that differentiation of primary and secondary muscle fibres will be completed by day 90 in the pig, as discussed in chapter 2. Therefore, even if the effects of uterine crowding on muscle fibre numbers are associated with decreases in fetal weight at day 90, subsequent increases in fetal weight will not correct the problem of limited development of muscle fibre number.

In Chapter 6, analysis of each litter in order to identify classical IUGR fetuses, based on those that fall two standard deviations below the mean body weight of the litter, revealed that runt fetuses were present in twelve of the litters from the crowded group, whilst only two of the litters from the ligated treatment group contain a runt pig. The information reviewed in Chapter 2 indicates that the growth potential of runt pigs within a litter will forever be compromised. Effects on muscle fibre development, created by maternal under-nutrition during gestation, resulted in lifetime limitations in growth performance and muscle mass. It is reasonable to assume that the effects of embryo crowding *in utero* on the number of secondary muscle fibres observed in Chapter 6 may be associated with similar limitations on postnatal growth performance. Whilst not all pigs with a high number of muscle fibres have fast and efficient growth rates, pigs with a low numbers of fibres invariably grow less well. As a higher number of muscle fibres is a prerequisite for the potential to grow well (Dwyer et al., 1993), mixing pigs during the production process that have been compromised by a crowded uterine environment early in gestation, with smaller weight pigs that were not the runts in their litters, will not compensate for differences in developmental potential. Postnatal nutritional intervention is also unlikely to alleviate the effect of reduced muscle fibre number, since potential for catch up growth depends on the presence of a high relative number of muscle fibres as discussed by Handel and Stickland (1988). In fact, as grow-finish diets are normally formulated to meet the needs of pigs with the highest lean growth potential, the overformulation of nutrients for IUGR-type pigs has important environmental implications. The dilemma from a practical aspect is that the less extreme IUGR effects are not even associated with reduced birth weight and cannot therefore be effectively recognized at farrowing.

The relative MHC isoform distribution of the ST muscle was examined in neonatal piglets (Chapter 3) and day 90 fetuses (Chapters 5 and 6). Western blotting was used to identify each of the four MHC isoforms (as described in Chapter 6) and the mean isoform distribution (%) was examined. From the perspective of effects of crowding *in utero* and IUGR on myogenesis, there were no differences in isoform distribution pattern in day 90 tissue between control and ligated animals in either Chapters 5 or 6, indicating the absence of qualitative differences in myogenesis. This was despite the fact that a higher level of uterine crowding resulted in a significant decrease in the number of fetal secondary muscle fibres in Chapter 6. In neonatal tissue, the embryonic MHC isoform was the most abundant followed by IIa and fetal MHC in similar quantities. Type Iβ MHC was present in the lowest quantity. In contrast, in day 90 fetal samples, the fetal MHC isoform was the most abundant, followed by embryonic, -IIa and -Iβ (Table 7.2).

Table 7.2 Myosin Heavy Chain Isoform distribution (mean % ± SEM) in neonatal (N = 23; Chapter 3) and day 90 (N = 28; Chapter 6) semitendinosus muscle.

	***Semitendinosus* Muscle Tissue**	
Parameter	**Day 90**	**Neonatal**
Embryonic MHC (%)	24.17 ± 1.31^{a}	38.35 ± 1.59^{b}
Fetal MHC (%)	57.04 ± 1.50^{a}	26.29 ± 1.36^{b}
Type IIa MHC (%)	12.96 ± 1.22^{a}	27.03 ± 1.33^{b}
Type Iβ MHC (%)	6.65 ± 0.78	8.33 ± 1.12
Developmental:Adult MHC isoforms	5.24 ± 0.69^{a}	2.11 ± 0.21^{b}

Means ± SEM within a row with different superscripts differ ($P < 0.001$)

Myosin isoform nomenclature has been well established and it does not follow that the embryonic isoform is developmentally "less mature" than the fetal isoform as would be expected from the traditional definition of embryonic and fetal stages of growth and development. Expression of embryonic and fetal isoforms is not restricted to the embryonic and fetal stages of development. Indeed, as discussed by Dunn and Michel (1997), the re-expression of developmental myosin isoforms, particularly embryonic MHC, within rapidly growing adult fibres has been linked to the process of satellite cell proliferation and the subsequent incorporation of myonuclei into existing muscle fibres. It is possible that the process of normal growth and development in neonatal tissue, as well as the increased activity level and weight bearing during the first day of neonatal life prior to slaughter, may be associated with satellite cell proliferation, explaining the associated increase in embryonic MHC in neonatal tissue.

Overall, the increased maturity of neonatal muscle compared to day 90 muscle is apparent when the ratio of developmental:adult isoforms is examined. Day 90 tissue contained a higher proportion of fetal and embryonic isoforms than neonatal tissue. These are the first data from our group on the distribution of myosin isoforms in neonatal pig ST muscle and establish the basal conditions for future comparisons of relative MHC isoform distribution.

An ongoing study of the effects of intrauterine crowding on development of skeletal muscle is presently being undertaken in the Master's program of Wai Yu Tse, to identify possible differences in the expression of some of the muscle regulatory factors between day 30 embryos from the control and ligated animals described in Chapter 6. *In situ* hybridisation and real time polymerase chain reaction (RT-PCR) techniques are being used as a qualitative and quantitative measure of muscle regulatory factor expression. Based on the finding of a decreased number of secondary muscle fibres in the control group of animals in Chapter 6, it is of interest to discover the time point during gestation when the effects of increased numbers of embryos *in utero* may detrimentally re-program the process of muscle fibre development.

In conclusion, the extent of uterine crowding that was created in the studies described in this thesis, using both gilts and higher parity sows, has been less than the crowding originally predicted in at least a sub-population of higher parity sows in existing commercial dam-lines. Nevertheless, differences in the prenatal environment had consequences for the pattern of muscle fibre development. Together with the literature reviewed in Chapter 2, these results provide a good insight into the possible biological origins of much of the postnatal variability in growth performance encountered in the swine industry. Consequences of more extreme crowding *in utero* on fetal and postnatal development, resulting from changing patterns of early embryonic survival, certainly merit further investigation.

The prenatal environment clearly effects development *in utero*, and evidence suggests this will make a profound contribution to postnatal variation in growth performance. In terms of practical implications for pork production, defining thresholds for effects of uterine crowding is a complex issue. Collectively, the four studies presented in this thesis indicate that the potential for uterine crowding to impact fetal development depends on an interaction between parity, genotype and the health status of the sow population. However, very limited data on each of these factors, let alone their interaction, are available.

The implications of these findings for the swine industry are widespread. Clearly major differences exist in the key reproductive characteristics of different commercial dam-lines. Very few swine genetics companies are aware of these traits, since knowledge of ovulation rates, and rates of embryo survival to day 30 necessitate expensive surgical intervention or killing of pregnant, productive animals. However, it is clearly important to identify specific dam-lines in which the imbalance between ovulation rate and embryonic survival, and uterine capacity, is a particular problem for fetal development. Herd depopulation exercises offer an opportunity to gather information from a large number of animals, although they are usually carried out for an underlying reason such as poor health status, which could affect reproductive parameters. As recently discussed (Foxcroft and Town, 2004), once reproductive traits are known, improved selection programs may be able to account for different components of litter size, and decrease variation in development and optimise lean growth performance of all piglets in a litter.

Evidence from these studies indicates that piglets from equal litter sizes at term, which may even have very similar birth weights, could have originated from different intrauterine environments in terms of numbers of embryos around day 30 of gestation, subjecting them to different patterns of fetal growth and development. Such observations are consistent with research discussed in Chapter 2, regarding the "fetal origins of adult disease hypothesis", whereby the occurrence of long term health effects following fetal undernutrition may occur in the absence of an effect on birth weight (as suggested by Lumey, 1998a). One practical application of this information may be development of a strategy to segregate pigs at weaning, based not only on birth weight, but also accounting for anticipated developmental potential (Foxcroft and Town, 2004). For example, avoiding mixing offspring from gilts with piglets from mid or higher parity sows. Clearly a better understanding of critical reproductive characteristics of existing commercial dam-line sows, could lead to benefits for commercial swine production.

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